Studies on the Structure and Function of Membranes of Nematohelminths

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BY

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CERTIFICATE

This is to certify that the work embodied in this thesis has been carried out by Shri Khalid Masood, M.Sc., M.Phil., under my supervision. He has fulfilled the requirements for the degree of Doctor of Philosophy in Biochemistry of the Aligarh Muslim University, Aligarh, regarding the nature and prescribed period of investigational work.

The work included in this thesis has not been submitted for any other degree, and unless otherwise stated is all original.

(V.M.L. SRIVASTAVA)

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Preface

Helminth parasites are pathogenic not only to the human race but also to the animals of wild and domesticated nature. At present nearly 300 million people are infected with filarial worms and every third person in India faces the risk of exposure to filariasis. Yet there is no chemotherapy which is both adequate and safe. The majority of infected individuals live at subsistence levels in village agricultural areas of developing countries where health is often closely related to economic survival; the incentive to control the disease is, therefore, both human and economic.

Nematode cuticle which serves as a barrier between its body and the surroundings plays an important role in the physiology of the worm. This is the site which at first is exposed to the attack of either the chemotherapeutic or the immunoprophylatic agents. Hence the understanding of biochemical nature of nematode cuticle becomes a subject of utmost importance Such information may be useful in devising rapid methods for the identification of ineffectiveness of drugs; in developing protective vaccines and improved immunodiagnostic reagents as well as in elucidating the nature of host-parasite interaction.

Present dissertation describes the transport of metabolites across the cuticle of Ascaridia galli, Litomosoides carinii, Dipetalonema viteae and Setaria cervi; extraction, purification and characterization of proteins/glycoproteins from the cuticular surface of A.galli and identification of carbohydrate moieties and their role in the mode of action of drugs on microfilariae.

ABBREVIATIONS

BFM	Basic Filarial Medium
BSA	Bovine Serum albumin
cAMP	Cyclic adenosine 3',5'-monophosphate
CD	Circular Dichroism
Con-A	Concanavalin-A
CSN	Carcinoscorpin
EDTA	Ethylene diamine tetra acetate
FITC	Flourescein Isothiocyanate conjugate
KRB	Kreb's Ringer bicarbonate
mCi	millicurie
mM	millimolar
m u	milli micron
nm	nano meter
n mole	nano moles
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCA	Perchloric acid
PNA	Peanut agglutinin
POPOP	Dimethyl 1,4 bis (2-(5-phenyloxazole)-benzene
PPO	2,5-diphenyl oxazole
RCA_1	Ricinus communis agglutinin
RCA_2	Ricin
SBA	Soyabean agglutinin
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
WGA	Wheat germ agglutinin
uM	micro moles
uCi	Micro curi

CHAPTER I

INTRODUCTION

Parasites are widespread in nature. They infect not only the man but also the birds and animals of his daily need. Among endoparasites, helminths, trichomonads and hemoflagellates are the most important groups of parasites. Helminths may further be subdivided as mematohelminths (nematodes and acanthocephala) and platyhelminths (trematodes and cestodes).

Existence of helminth parasites was in the knowledge of the people of many countries even since the dawn of human history. In India, Atharva-Veda describes such infections with the general term 'worms' (Jolley, 1951). The ravages caused by various infections are now well known. The pathogenicity of parasites varies greatly depending on their number, habits, nature of imigration and, particularly the degree of adaptation that has developed between the host and the parasites.

Worms frequently cause a marked eosinophilia. This is often seen when the host has developed a certain degree of resistence to the parasite. Among other common symptoms are the anemia and hemorrhage, which may be due to the loss of blood by sucking, the destruction of erythrocytes or the secretion of anticoagulants. Degeneration and death of cells occurs when the parasites either invade the cells or when they feed on the tissues of the host. Mechanical obstruction of hollow organs has been described as a general feature of helminthiasis e.g., of cardiac chambers in dogs by Dirofilaria immitis, of bile ducts of cattle and sheep by flukes of intestines by ascaris and, of human lymphatics by filarial parasites.

Schistosomiasis is a parasite-disease caused by trematodes living in the blood. Today, it is one of the major tropical diseases that affects 200-300 million people in Africa, Asia, South America and Caribbean islands (Gentilini et al., 1977). Hookworm disease (Ancyclostomiasis) occurs in 20-25% of the world population, primarily in agricultural areas where soil temperatures between 23 to 33^{0} (Davis, 1973). In tropical countries, 70-90% of the population is estimated to be infected with Ascaris lumbricoides (Warren and Mahmoud, 1978). Just like ascariasis and the hookworm, trichuriasis is also a soil transmitted nematodal disease. The infection is cosmopolite and very often occurs in institutions for the mentally insane.

The other most devastating disease of the world is filariasis. At least 20 million people in tropical Africa, Yemen, Mexico and Central and South America are infected with onchocerciasis, the disease commonly known as River-blindness. Fortunately, in India onchocerciasis is rarely found. However, the other type of filariasis, caused by the lymphatic dwelling parasites, Wuchereria bancrofti and Brugia malayi. These filariids infect about 250 million people in West, Central and East Africa, Egypt, Madagascar, South-East Asia, China and Phillipines.

Improvements in enviromental sanitation and hygienic conditions have undoubtedly led to the eradication of many infectious diseases in Europe and America. However, in tropical countries, like India, where sanitary conditions and environment and a low standard of living are conducive to the preservation and spreading of parasites, worm infections are one of the major problems. Although most of the diseases are not fatal however, in acute cases the patients become physically unfit. It accounts for tremendous loss of man hours which a developing country, like India, can ill afford to incur. The World Health Organisation has, therefore, included schistosomiasis and filariasis among six groups of diseases (WHO, 1982).

As mentioned above lymphatic filariasis has become a disease of great concern from our national point of view also. In 1958, 65 million Indians were exposed to

filariasis. The figure rose to 125 million in 1969 and 236 million in 1976 and today every third person faces the risk of exposure to the disease (Subrahmanyam, 1982). Filarial infections are generally chronic and often disabling. Local and systemic sensitization and tissue reactions to the invader may primarily be held responsible for the development of these symptoms. Hyperplasias and the accumulation of host cells later construct and block the lymphatic vessels causing painful swelling. In extreme cases, the infection leads to gross wellings of the extremities, scrotum or breast. This is what has given the disease its more popular name elephantiasis. Swollen legs as a symptom had been recognized by the Persian Physicians around 600 BC. The famous Indian Physician Susruta has recorded its occurrence in India in his noted classic Susruta samhita around AD 200. He describes the elephantoid legs as shilpadan, which means stone legs.

Although the symptoms were recognized long back, the cause of the disease was discovered only about one century ago. The microfilariae were first seen in 1863 by a Frenchman M. Demarquay in a patient's hydrocele fluid in Havana, Cuba. In 1866, O. Wucherer, a German scientist, found an unknown worm in a Brazilian which was later identified to be that of filaria. Thereafter in 1872, T.R. Lewis identified filarial parasite in the peripheral

blood of a patient in Calcutta. In 1874, an Italian scientist P. Sonsino described a similar parasite in the blood and urine of some Egyptians. Soon after Joseph Bancroft discovered the adult female parasite in the lymphatic abscess of a Chinese patient. Thomas Spencer Cobold linked this parasite to filariasis and named it Filaria bancrofti. A year later, Lewis firmly established the link and he described it as Filaria sanguinis hominis.

In 1878, at Amoy in China Patrick Manson, known as the father of tropical medicine saw a parasite at larval stage in the flight muscles of a mosquito. He claimed it Filaria sanguinis hominis, equipped for independent life, ready to quit its nurse, the mosquito.

The parasite, in honour of the German scientist, was later remained as Wuchereria. Such were the series of events that heralded the beginning of our knowledge of the fascinating life cycle of the filarial parasite.

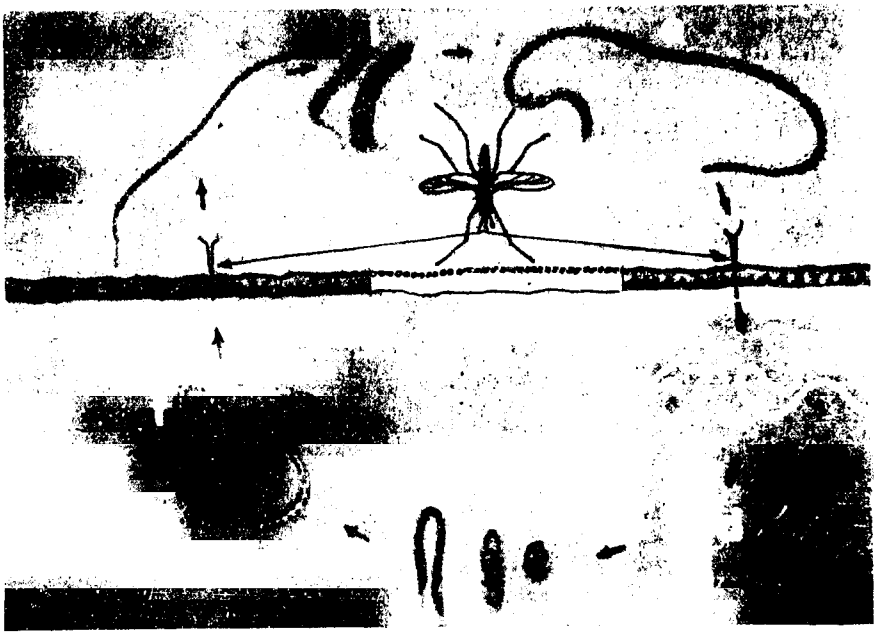
Now we know that filariasis is caused by tissue dwelling parasitic worms which are transmitted from man to man through intermediate insect hosts, mosquitoes, midges and flies. The thread like filarial worms, belonging to the superfamily Filarioidea contains well over 500 species. The adults of this group are parasitic in the circulatory system, lymph system, muscles, connective tissues or scrous cavities of veratebrates, whilst the

intermediate host is always an arthropod. Only seven species of filarial worm are known to infect man. These are Wuchereria bancrofti, Onchocerca volvulus, Brugia malayi, Loa loa, Tetrapetalonema (=Dipetalonema) perstans, T. streptocerca and Mansonella ozzardi. Of these, the first two are the most common pathogens. Man is also occasionally infected with the dog heart worm Dirofilaria immitis.

The life cycle of the parasite is complex. It goes through three development stages first as the microfilaria, next as the infective larva and finally the adult worm. Figure 1 depicts the life cycle of the lymph-dwelling filarial worm, Wuchereria bancrofti.

As discussed above, helminth parasites are pathogenic not only to the human race but also infect the animals of wild and domesticated nature. Symptomatically it is hard to describe the pathogenesis of the disease in animals. However, histochemical and electron microscopic examinations have revealed the tissue damages resembling closely to those found in humans. For example, Filaroides milksi in dogs cause thickening of pulmonary pleura and a loss of elasticity of the lungs, which become very dense and contain firm grey airless masses with large white internal foci (Jubb, 1960). Emphysema between the solid portions of lung has also been observed. Mature

Fig. 1. The lifecycle of a filaria parasite: (a) the infective larvae, known as the third-stage larvae, migrate to the mouth-parts of a mosquito and infect man when it bites; (b) the infecting larvae head for the lymphatics of human host and mature in about a year; (c) the thread-like adult can live inside the host for many years; (d) the blockage caused by the worms may lead to elephantiasis; (e) the females produce enormous number of larvae each day; (f) these mature into microfilariae; (g) the microfilariae are picked up by the female mosquito while it feeds; (h) in the mosquito, the microfilariae penetrate through the stomach walls and (i and j) they metamorphose in the thorax muscles to the third-stage larvae in about 10 days. After this the cycle repeats.



viviparous parasites are found in the bronchioles and also in the parenchymal tissue and the microfilariae are seen in the terminal bronchioles as well as in parenchyma. The main tissue reaction to the worms has been described as the granulomatous which may be a response to the presence of microfilariae.

Healthy livestock comprise one of the man's most valuable economic resources. They provide high quality of edible proteins, fibres of all types and enormous amount of useful by products such as leather, horns etc. and in the developing countries above all, motive power and fuel (Kelley and Hall, 1979). In order to maintain an adequate supply of such products for the increasing human population, it has been estimated that the efficacy of ruminant production will have to be increased at least by 50% over next two decades (Byerly, 1977).

It is pertinent to mention that in the Second International Congress of Parasitology (1970) attention has been drawn towards the economic and other aspects of parasitic diseases among domesticated animals. The existence of the problem has been given recognition and two points were considered of prime importance, the method of diagnosis, particularly at subclinical level, and the economic benefits of antiparasitic treatment.

Inspite of the extraordinary progress made over the last decade there are many facets of anthelmintics

modification still in need of improvement. The first and most important of these is the development of cheaper drugs, and the other is the discovery of compounds with wide range of activity.

One of the primary requisite for chemotherapeutic agent is its selective toxicity for the parasite compared to its low toxicity towards the host. Therefore, an understanding of the physiological and biochemical differences between host and the parasite is of utmost importance (Saz and Bueding, 1966). Such differences provide opportunities to design chemical agents that interfere with the functional integrity of the parasite without injury to the host. The chemical constituents of the surface leading to the disguise of the antigenic determinants may also be exploited for developing suitable immunotherapeutic measures.

It should be realized that there is no dearth of such functional differences between the host and the parasite because, parasitism is not a degradation rather is a specialization. The parasites, in accordance of their environment, have undergone great morphological changes. It is represented by the loss of unnecessary organs, like eye spots in internal parasites, the loss of wings in certain external parasites and even of the alimentary canal in tape worms and acanthocephala. Special development in the organs of adhesion such as hooks and suckers, and

the organs of reproduction are the examples of the appearance of new organs as an adaptation to their mode of life.

This adaptation is ought to be reflected at the biochemical and genetic level and offers interesting sites for wanted specificity of chemotherapeutic agents. For example, studies on the carbohydrate metabolism have shown that helminth parasites, unlike their vertebrate hosts, do not completely oxidize sugars into carbon dioxide and water even in presence of adequate supply of oxygen. Accordingly most of the known antihelmintics inhibit carbohydrate metabolism of the parasite but do not effectively block the metabolism of the host (Saz and Bueding, 1966). Many more pathways and sites need extensive digging in order to approach chemotherapy and immunology in a rational way. According to Baldwin (1948), although comparative biochemistry of parasites has emerged from its infancy, it still lags behind to the study of the biochemistry of vertebrates.

Cuticle or the outer body covering plays an important role in the life cycle of an organism. For example, it protects the parasite against the defence mechanisms of the host such as, enzymatic digestion in case of intestinal parasites or the immunological response in case of blood and tissue dwelling parasites. Cuticle also acts as a barrier in the transport and absorption of

nutrients and growth promoting factors. Chemoreceptors and neurotransmitters present on the surface mediate the processes leading to the adaptation in response to the enviromental stresses. Recognizing the importance of the cuticle, there has been an increasing demand for information about its role in nutrition, the penetration of anthelmintics, immunology, locomotion and molting. Development of immunotherapeutic measures against the surface constituents of parasites in recent years has made the study of the chemical nature of the parasitic surface more and more important. Availability of sophisticated instruments and development of newer techniques has now made it possible to look at the surface in professionally better way.

Unavailability of human parasites in sufficient quantities has always been a handicap to undertake such investigations. To overcome this problem, nematode parasites of the animal origin have to be used as models for generating the desired information.

In the present study a glycoprotein from the cuticular surface of Ascaridia galli (fowl intestinal nematode) has been purified and well characterized. The cuticle of this nematode has also been examined for the uptake of various amino acids through it. Filarial parasites - Setaria cervi, Litomosoides carinii and Dipetalonema viteae which are available in very small

amounts have been subjected to the specific studies only. Thus microfilariae were used for the analysis of nature of sugar moieties on their surface employing lactins as tool, while the adult worms were investigated for the transport of some important compounds e.g., methylglucose, cAMP and isoproterenol.

CHAPTER II

SURVEY OF LITERATURE

Present state of knowledge on the structure and composition of the helminth cuticle has resulted from the application of electron microscopy and other modern biological techniques. Available evidences indicate that the three main groups of helminths, the platyhelminths, nematode and acanthocephala, have quite different external coverings. The cuticle of digenetic trematodes and cestodes seems to be syncytical cytoplasmic layer which is continuous with nucleated cytoplasma situated in the parenchyma underneath the musculature of the body wall. The surface of tapeworms is formed into microvilli which increases the absorptive area. The most superficial layer contains mitochondria and enzymes which are believed to be concerned with active transport of substances. Microvilli do not occur in the digenetic trematodes but vacuoles and vesicles, mitochondria and enzymes do occur

in the outer most layer of the body and these may serve the same purpose of transportation. In the Acanthocephala, pores and canals in the cuticle and body wall are presumed to be conduits for nutrient materials passing into a body which, like that of cestodes, lacks an alimentary canal. On the other hand, the cuticle of nematodes has a very complex and varied structure.

The body wall of nematodes consists of an external cuticle, a hypodermis and a single layer of longitudinal muscle cells (Fig. 2). The cuticle covers the whole of the external surface and also lines the buccal cavity, oesophagus, rectum, cloaca, vagina and excretory pores. Nematodes owe much of their success as a large and ubiquitous group to an organisation which includes a cuticle of great ultrastructural complexity and evolutionary plasticity. Much more is known about the structure and composition of cuticle of nematoda than about that of other helminths.

The literature on this subject shall be discussed under the following heads and subheads:

1. Structure

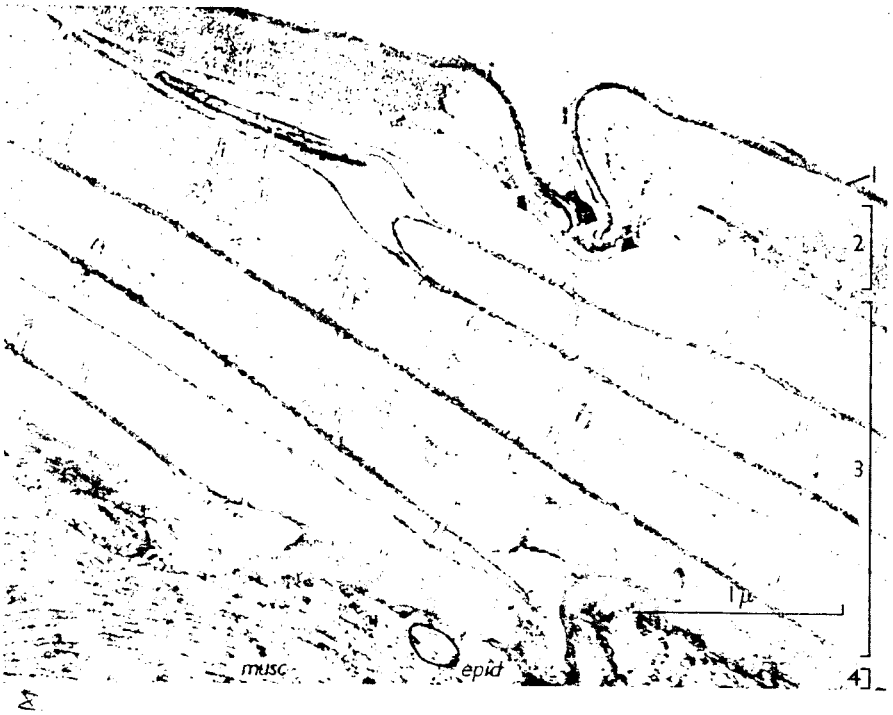
a) Ultrastructure

b) Histochemical

c) Chemical composition

d) Biochemical nature of the cuticular surface

Fig. 2. Electron micrograph of the cuticle of the anterior region of Euchromadora vulgaris (longitudinal section) to show the four major layers and the canals in the middle layer. 1, Outer membrane; 2, cortex; 3, middle layer formed from a series of overlapping plates; 4, a basal layer. epid, Epidermis (hypodermis); muse, muscle.


1
2
3
4
1μ
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epid

2. Functions

a) Protective

b) Immunological

c) Transport and permeability

d) Locomotion

1.a. Ultrastructure

The cuticle of nematodes is basically a three layered structure with an outer cortex, a middle matrix and an inner basal layer (Fig. 2). These three layers which are of varying thickness in different species, are often subdivided due to which it sometimes becomes difficult to determine their boundaries particularly in electron micrographs. Furthermore, since several of the layers of the cuticle are fibrous differing only in the orientation of the fibrils or degrees of compactness, it may even be difficult to assign any one layer as a particular basic layer. Inglis (1964a,b) considered that the three layered cuticle is modified around a canal system, believing that the punctations in the cuticle of the chromadorida and other nematodes represent canals. Though such canals are found to occur in Ascaris lumbricoides, Stronglus equinus and Euchromadora vulgaris (Bird, 1958a; Watson, 1965a,b), however, their occurrence may not be generalized. Presence of fibrils to a greater or lesser extent in the cuticle appears to be a common

feature for nematodes. In some nematodes fibrils are difficult to resolve, even with the electron microscope, and these layers then appear homogenous. On contrary, in some parasites fibrils are compacted basically to form two or three layers of fibres, e.g. in Ascaridoidea (Inglis, 1964a). In others (Enoplus, Dosylaimus and Mermis) the fibrils in the outer part of the matrix form fibres, while in Oxyuris equi, the fibrils form layers of fibres in the outer part of the matrix layer and of the basal layer (Bird, 1958a). This formation of fibres from the layers of fibrils is usually, but not always, associated with an increase in size of the body and may have mechanical associations in locomation (Harris and Crofton, 1957). According to Watson (1965a) the struts in the fluid filled layer of the cuticle of adult N.brasiliensis which support the longitudinal ridges of the cuticle may, originate from rod like structures similar to those found in the matrix layer of some free living marine nematodes.

1.b. Histochemical

The nematode cuticle has been traditionally viewed as an acellular exoskeleton. Chemically, its major structural elements are proteins similar in many respects to collageons, which are ubiquitous components of

extracellular connective tissues (Lee, 1966; Bird, 1971). In many cases it appears to be separated from the hypodermal cytoplasm by a membrane (Watson, 1965; Roggen et al., 1967), and is itself devoid of cellular organelles. However, the outermost boundary of the cuticle is a trilaminate layer approximately 100A° thick. This raises the possibility for the cuticle being limited by a plasma membrane, and thereby to be considered a cellular derivative (Bird, 1971). On the other hand, many insect cuticles are covered by a morphologically similar structure, referred to as "cuticulin", which per se is not a cytomembrane, but a stabilized layer of secreted lipids (Fisher, 1971). The electron microscopic studies thus support the early view of Chitwood and Chitwood (1950) that the nematode cuticle is derived internally from part of the hypodermis, rather than as an extracellular secretion therefrom. Thus the cuticle might be interpreted as a modified cellular component. According to Bonner et al. (1970), the outer boundary appears to be a plasma membrane derived from the apical hypodermal membrane.

This membrane is directly formed from cytomembranes of the endoplasmic reticulum, while the internal layers of the cuticle form by differentiation of the hypodermal cytoplasm. The process in certain respects appears to be analogous to the cytomorphosis manifested in the superficial

layers of the mammalian epidermis, where cells originating in the basal regions undergo keratinization as they are displaced toward the surface.

The keratin synthesized within the cytoplasm of these epidermal cells eventually displaces the organelles, which atropy, though the original plasma membrane is retained unlike the keratinized epidermal cells of mammalian skin. However, the nematode cuticle remains metabolically active, possessing enzymes, RNA, and the capability for further growth and differentiation (Lee, 1962; Anya, 1966a,b; Watson, 1965). As emphasized by Bonner et al. (1970), this is accomplished by the maintenance of an intimate relationship between the cuticle and hypodermal cytoplasm, manifested structurally in at least some nematodes by discontinuties in the cuticle-hypodermal membrane. The hypodermis of most adult parasitic nematodes is syncytical, with the nuclei located in the lateral cards. As determined by Thust (1968), the hypodermis of Ascaris lumbricoides is derived from the blastomeres which form a layer of discreet cellular units merge to form a syncytium. The hypodermal cytoplasm of these and other nematodes contains glycogen, lipid deposits, mitochondria, ribosomes, endoplasmic reticulum and golgi bodies (Wright, 1968; Jenkins, 1969; Hinz, 1963; Wisse and Daems, 1968; Lee, 1966b; Lee, 1970; Bonner et al., 1970; Kozek, 1971). During moulting, the

endoplasmic reticulum hypertrophies in connection with synthesis of cuticular proteins (Lee, 1970; Bonner et al., 1970).

1.c. Chemical composition

Bird (1954, 1956, 1957, 1958a,b) carried out a thorough and extensive study into the chemical composition of the cuticle of nematodes. He found that the sheath of the third stage larvae (i.e. the uncast cuticle of the second stage larvae) of Oesophagostomum, Ostertagia, Chaberila, Haemonchus and Trichostrongylus is soluble in water at 105°C. Nine amino acids were identified in hydrolysates of mixed collections of larval sheaths and in pure samples of sheaths from H.contortus. These amino acids, in order of the amounts present, were proline, hydroxy proline, aspartic acid, cysteic acid, glutamic acid, alanine, leucine, glycine and valine. Tests for cystine and tyrosine gave negative results (Bird, 1954).

Bird and Rogers (1956) found collagen in the cast sheaths of larval Trichostronglus such as Haemonchus and, Simmonds (1958) found collagen in the cast cuticle of the fourth stage larva of Nippostronglus brasilensis. Bird and Rogers (1956) were unable to demonstrate a tanning mechanism in the sheath of these larvae. However, Monne (1959), who described the larval cuticle of Dictyocaulus as collagenous, claimed that quinone tanning occurs in the

cuticle of these larvae and that the larval cuticle is more resistant to the action of probably proteolytic enzymes than is the adult cuticle. Savel (1955) identified thirteen amino acids in the cuticle of A. lumbricoides. He drew attention to the ratio of histidine, lysine, arginine (1:5:3) which is close to the ratio in many keratins (1:4:121:1:5:15) and considered that the cortex is composed of a keratin. This layer is however, soluble in hot dilute alkali, which is strong evidence against it containing keratin (Fairbairn, 1957). The cuticle of A. lumbricoides contains about 75% of water and small amounts of carbohydrates and lipids, as well as the predominating proteins (Fairbairn, 1956, 1957; Fairbairn and Passey, 1957). Apparently, the cuticle of nematodes is composed of a secreted collagen associated with hyaluronic acid, chondroitin sulphate containing acid mucopolysaccharides and a small amount of lipid. The collagen is usually present as fibrils which are more numerous and more closely associated with each other in some layers (the cortex and the fibre layer) than in others (the matrix layer). The outer cortex contains more sulphur than is found in other layers. This probably is due to disulfide linkages which, together with another type of chemical bond, stabilize the outer cortex and not due to keratin. Polyphenol-quinone tanning also plays

some part in stabilizing the outer cortex of nematodes but not all of them. The cuticle may contain a number of enzymes, RNA and hemoglobin and is evidently not an inert covering.

1.d. Biochemical nature of the cuticular surface

The way cells react to their immediate surroundings is in several respects dictated by surface carbohydrates. Membrane glycoproteins and glycolipids have been shown to be implicated in a wide range of phenomenon such as cell recognition, transport of solutes, hormone and drug receptors etc. (Lennarz, 1982).

Lectins, a class of carbohydrate-binding and cell-agglutinating proteins of non-immune origin, provide a powerful tool to the isolation and characterization of soluble glycoproteins and for probing cell surface sugar moities (Reisner and Sharon, 1980). However, only recently few investigators have directed their attention to this important subject in parasitic biochemistry. Hemoflagellates (Trypanosomes and Leishmania) have been well studied regarding their surface carbohydrates compared to helminth parasites. Among the latter class, only Schistosoma mansoni has received sufficient attention. Thus, Simpson and Smithers (1980) reported that adult male S.mansoni had very high affinity for concanavalin-A and Ricin communis agglutinin; while the parasite bound poorly to

wheat germ, soyabean and peanut agglutinins. This indicated the presence of glucose, mannose and N-acetyl-glucosamine in high concentrations on the worm surface. Friedman et al. (1982) in Spirometra mansonoides identified tegumental glycopeptides by the analysis of lectin. Glycopeptides of brush border membrane were identified by the direct application of the following flourescein isothiocynate-conjugated lectins to slab gels: Concanavalin-A, wheat germ agglutinin, Ricinus communis agglutinin-120, soybean agglutinin, and Ulex europaeus agglutinin-1. Based on the different sugar specificaties of the lectins tested, the oliogosaccharide chains of tegumental glycoproteins of S.mansonoides was suggested to contain: D-mannose, D-glucose, N-acetyl-glucosamine, N-acetylneuraminic acid, D-galactose, and N-acetyl-D-galactosamine. Investigations on the metabolic events associated with the synthesis of glycoproteins, oligosaccharides and glycolipids in adult worms of S.mansoni (Rumjanek and Smithers, 1978) revealed the occurrence of a membrane bound enzymatic systems which was able to transfer mannose from guanosine diphosphate-mannose to a chloroform soluble compound, forming a lipid linked oligosaccharide. Homogenate of this parasite also transferred glucose and galactose from their uridine diphosphate derivatives to a lipid acceptor, in comparison

fucose and glucosamine were poorly transferred. The lipid acceptor was believed to be an intermediate in the glycosylation of the worms glycoproteins and glycolipids. Since glucose, mannose and galactose were the major monosaccharide components of the worms tegument, Rumjanek et al. (1979) suggested that the mechanism of glycosylation of tegumental macromolecules may occur through the glycosyl transfer system.

Surface carbohydrates of different stages of Brugia malayi, (the human filarial parasite) viz., microfilariae, infective larvae and adult worms were analyzed by incubating a panel of flourescinated lectins (Kaushal et al., 1983). They found that infective larvae and adult worms did not bind significantly any of the lectins, while the microfilariae bound wheat germ agglutinin. The binding of this lectin was found to be saturable and specific, thereby showing the presence of N-acetyl-D-glucosamine on the microfilarial surface. In addition, in vitro released microfilariae bound concanvalin-A indicating the presence of glucose/mannose on this form of the parasite. However, similar concanavalin-A binding was not observed with in vitro released microfilariae. It was attributed that the masking or loss of surface components during development of microfilariae occurred in vivo.

Furman and Ash (1983) found that the sheath of mature *in vivo* derived *Brugia pahangi* microfilariae bound concanavalin-A and wheat germ agglutinin, thereby indicating the presence of N-acetyl glucosamine and glucose or mannose. The sheath of immature in utero-derived microfilariae also bound *Limulus polyphemus* agglutinin, peanut agglutinin, *Ricinus communis* agglutinin-I, and soyabean agglutinin, thus indicating the presence of the additional sugars like galactose, sialic acid and N-acetyl galactosamine. However, none of the tested flouresecinated lectins bound to either mature or immature exsheathed microfilariae of *B.pahangi*.

2. Functions

2.a. Protective

The cuticle of nematodes has several functions, notably to protect the parasite against the mechanical and chemical injury directed by the external surroundings thereby allowing the worms to regulate their internal environment. As a result, they have been able to invade almost every type of ecological niche. For this important functions, the parasites equip themselves with some special weapon e.g. Ascarid's cuticle possesses and secrets into the surroundings antienzymes in the form of trypsin- and chymotrypsin inhibitors (von Brand, 1973).

As discussed below blood and tissue dwelling parasites absorb host derived materials, proteins in particular, to protect them against the immunosurveillance of the host (Soulsby, 1971).

2.b. Immunological

The cuticle of several nematode species are thought to be antigenic (Crandall et al., 1963; Taffs and Voller, 1963; Baratawidjaja et al., 1963) and accordingly antibody binding to the outer surface of the cuticle has also been demonstrated (Crandall and Avean, 1967). However, data presented by Hogarth-Scott (1968) suggest that immunoglobulins adsorbed by certain nematode species represent cross reacting naturally occurring antibodies, rather than nematode specific antibodies. As reviewed by Soulsby (1971), several workers have shown cuticular binding of complement as well as antibody, resulting in the adherence of host blood cells, though the relationship of these phenomena to protective immunity remains undermined. Soulsby (1971) pointed out that the attachment of host serum proteins might actually benefit the parasite in that, the coating of such material would render the cuticular surface more 'host like' and thereby masked from the host's immunosurveillance mechanisms. The surface of certain nematodes bears a density of electronegative charges (Hudson and Kitts, 1971;

Bonner et al., 1970) which may reflect the presence of acidic glycans. Such materials are themselves, generally, weakly antigenic (Apfel and Peters, 1970). Treatment of Protostrongylus larvae with neuraminidase reduces this surface charge (Hudson and Kitts, 1971) and concomitantly facilitates attachment of host leukocytes to the cuticle. This observation suggests that the electronegative charges on the cuticular membrane may serve as a physical barrier to cell contact, in that leukocytes possess a similar surface charge, or that the neuraminic (sialic) acid residues otherwise militate against immunogenicity of the cuticular membrane (Apfel and Peters, 1970). In the last two years immunology of cuticle of helminth parasites has dramatically advanced. Now monoclonal antibodies have been used as valuable tools in characterizing the antigens of schistosomes, and in studying the role of the surface antigens of the parasite in the killing of challenge infections (Taylor and Butterworth, 1982). Recently Baschong et al. (1982) have extracted and tested the immunogenicity of cuticular antigens from female worms of Dipetalonema viteae. Surface specific antigens were obtained by proteolytic digestion with proteinase K, or therolysin or subtilisin. When Golden hamesters were injected with extracts, interesting observations were recorded. Subtilisin and

thermolysin extracts provoked antibody formation against somatic structures (e.g., gut, uterus, muscles) but not against the cuticle, whereas immunization with the proteinase-K extract induced antibodies exclusively against cuticular hypodermic structures of female and male worms.

2.c. Permeability and Transport

Nematohelminths have well defined digestive system and may ingest food material from surrounding host fluid and hence need not to depend for uptake of nutrients through the body surface. Possibly due to this reason not much attention was paid towards the transport mechanisms of nematode cuticle. Although these parasites are known for long to osmoregulate through cuticle (Pannikar and Sproston, 1963) indicating the ability to transport ions. Furthermore a size of literature supports the concept that nematode cuticle is impermeable to low molecular weight polar solutes (Pappas and Read, 1975). The subject had been a matter of great controversy till the employment of electron microscopy in parasitology. Using this techniques the cuticle of Mermis migrescens, which was long known to be permeable to vital dyes (Chitwood and Chitwood, 1974) was later shown to possess morphological features consistent with the absorptive role of the body surface (Poiner and Hess, 1977). Since then number of reports have described absorption through the cuticular

surface. Thus, Weatherly et al. (1963) have shown that A.galli whose openings were closed with colloidin, absorbed alanine and glucose through the cuticle. Later, transcuticular absorption of various amino acids by Ascaris suum was also established (Berdyeva and Dryuchencko, 1972).

An alternative method to investigate transport mechanism was described by Harris et al. (1972) who demonstrated active transport of leucine through isolated cuticle of A.lumbricoides. Amino acids and sugars were reported to be transported through the gut of A.suum (Read, 1966; Castro and Fairbairn, 1969) and Trichuris vulpis (Bueding et al., 1961). Movement of lipids across the intestine of A.suum had also been reported (Beams et al., 1974). There are also reports regarding the permeability of antihelmintics through the Ascaris cuticle (Weatherly et al., 1963).

As far as the uptake of nutrients by adult filarial worms are concerned, presence of erythrocytes have been demonstrated in the digestive tract of Dirofilaria immitis (Maki et al., 1982) and the uptake of trypan blue by B.pahangi has been demonstrated in vivo (Howells and Chen, 1981), although in vitro B.pahangi does not ingest trypan blue or other high molecular weight substances (Chen and Howells, 1979a). Adult D.immitis similarly failed to

take-up trypan blue during *in vitro* incubations (Chen and Howells, 1981a). Adult filarial worms appear to be able to take up nutrients via cuticle *in vitro*, which together with the hypodermis shows structural modifications (Howells, 1980). Adult *B.phangi* can take-up D-glucose, L-leucine, glycine, cycloleucine and adenosine by a transcuticular route (Chen and Howells, 1979a,b; Howells and Chen, 1981; Nduka and Howells, 1980), whilst *D.immitis* has been shown to take up D-glucose and adenosine (Yanagisawa and Koyama, 1970; Chen and Howells, 1981a). The transcuticular uptake of glycine has also been demonstrated in *Onchocerea gutturosa* (Howells, 1980). In addition, Chen and Howells (1981b) have shown that adult *B.pahangi* can take up uracil, adenine, hypoxanthine and guanosine (but not thymine, cytosine, orotate, formate, p-aminobenzoic acid or folate) during *in vitro* incubations, whilst adult *D.immitis* can take up uridine and uracil (Jaffe *et al.*, 1972). Cuticular transport in *B.pahangi* and *D.immitis* is selective, and neither nematode, for example, takes up exogenous L-glucose, sucrose or thymidine (Chen and Howells, 1979a,b; 1981a,b). The transcuticular uptake of glycine and cycloleucine by adult *B.phangi* seems to take place by diffusion (Nduka and Howells, 1980). Though uptake of glucose and other monosaccharides from the incubation media has been shown by adult *C.hawkingi*,

L.carinii and S.cervi, the site of uptake was not investigated (Bueding, 1949a; Anwar et al., 1975; 1978; Srivastava and Ghatak, 1974; Srivastava et al., 1968).

The microfilariae of L.carinii and S.cervi have also been shown to take-up glucose from the incubation media (Rathaur et al., 1980). Microfilariae have a non functional gut and so uptake presumably takes place across the cuticle, although there are suggestions that the microfilariae of Breinlia sergenti ingest nutrients via the buccal cavity (Howells, 1980). The microfilariae of D.immitis can utilize exogenous glucose, amino acids and RNA precursors viz., uracil, uridine, adenine, adenosine but not thymidine (Jaffe and Doremus, 1970; Ando et al., 1980). Chen and Howells (1981b) have shown utilization of exogenous glycine, uracil, adenine, hypoxanthine and guanine but not thymine, cytosine, orotate, formate, p-aminobenzoic acid or folate by B.pahangi microfilariae.

The 3rd and 4th stage larvae of B.pahangi have been shown, in vitro, to take up glucose and a range of amino acids and nucleic acid precursors, presumably via a transcuticular route (Chen and Howells, 1979a,b; 1981b).

The adult and larval stages of filarial worms all appear, at least in vitro, to be able to take-up low molecular weight nutrients via cuticle. Thus the old

concept regarding the unabsorptive role of the cuticle appears no more valid, However, many more studies on the subject are required to finally disapprove the decades old view.

2.d. Locomotions

Another important function of the cuticle is related to locomotion. The cuticle imposes a restraint on changes of bodily shape, but its structure is such that restraint produces those changes in shape that can and must be made (Clark, 1964). Its elastic properties or mechanical interactions between its substractural components providing an antagonistic force to muscular action (Harris and Crofton, 1957; Inglis, 1964; Wisse and Daems, 1968). According to recent concept many antihelmintics act against the gastrointestinal parasites, particularly Ascaris, by causing a reversible paralysis of their neuromuscular system. Similarly tetramisole also causes spastic muscle paralysis in a number of nematodes including filarial parasites. This movement enables the intestinal parasites to maintain their position against the parastalitic movement of the intestine.

CHAPTER III

MATERIALS AND METHODS

Parasitic material

Ascaridia galli was obtained from fowl intestines collected from a local abbatoir. The worms were washed several times with normal saline in order to make them free from adhering host materials.

Half an hour prior to the commencement of various studies, the worms were kept in Kreb's Ringer bicarbonate buffer, pH 7.4 (KRB) DeLuca and Cohen, 1964) for bringing them in equilibrium with the ionic fluxes of the ambient medium.

Setaria cervi (bovine filarial parasite) was also obtained from a local abbatoir. The worms were brought to the laboratory in KRB supplemented with 1.0% glucose and generally used within 4-5 hr of their collection.

Litomosoides carinii and Dipetalonema viteae were isolated respectively from the pleural cavity of cotton rats and subcutaneous tissues of Mastomys natalensis maintained in this Institute. Both infections were 90 days old. The parasites were freed of host material by several transfers through basic filarial medium (BFM) (Bueding, 1949) containing 1% glucose. The average sizes of the male and female worms were 10 and 4 cm for L.carinii, and 9 and 3.5 cm for D.viteae respectively.

Microfilariae

Microfilariae from the heparinized blood of heavily infected hosts (Mastomys natalensis and cotton rat) were separated by filtration through 5 uM filters. BFM containing 1% glucose was used for the washing and the recovery of the microfilariae from the filters.

Chemicals and Reagents

^{14}C-labelled amino acids and ^{3}H-cAMP were procured from the Isotope Division, Bhabha Atomic Research Centre, Bombay. 3-Methyl-D-glucose-^{14}C-(U), ^{3}H-isoproterenol and hymine hydroxide were obtained from New England Nuclear, Boston, U.S.A. A kit of unlabelled amino acids was obtained from Loba Chemie, Bombay. Collodion was the product of Riedel-Hannover, Germany. cAMP, isoproterenol, phloridzin, lysozyme, aldolase and bovine serum albumin

were purchased from Sigma Chemical Co., St.Louis, U.S.A. Trypsin and papain were the products of Serva Chemicals, West Germany.

Acrylamide, methylene bisacrylamide, tetramethyl-ethylene diamine (TEMED), coomassie brilliant blue, sodium dodecyl sulphate (SDS), cAMP, diphenyloxazole (PPO) and 1,4-bis-2,4-methyl-5-phenyl oxyzolylbenzene (POPOP) were procured from SISCO Research Laboratories, Bombay. Concanavalin-A (Con-A), Ricinus cummunis agglutinin (RCA_1), Ricin (RCA_2), Peanut agglutinin (PNA), Soybean agglutinin (SBA), wheat germ agglutinin (WGA), Carcinoscorpin agglutinin (CSN) and Agarose bound Ricinus cummunis agglutinin were purchased from Hy-Gro Chemicals, Calcutta.

Diethylcarbamazine (DEC) was obtained as gift from Cynamid India Ltd., Bombay while Centperazine was synthesized by the Medicinal Chemistry Division of the Institute. Triphenyltin chloride and p-hydroxymercuribenzoate (pHMB) were obtained as gifts from the Biochemistry Department of the University of Notre Dame, South Bend, U.S.A. All other chemicals were of analytical grade and were available locally.

Membrane filters (5 uM) were obtained from the Maxflow, Bombay.

Extraction of surface constituents

Intact adult A.galli worms were incubated for half an hour at 37°C in Tris-buffered saline, pH 7.4 containing 20 mM EDTA, 50 mM Cysteine and papain (0.5 mg/gm worms). After removing parasites, the medium was acidified with perchloric acid (PCA) and centrifuged at 1,000 g for 10 minutes. The supernate was neutralized with alkali (KOH 60%) and centrifuged again. This supernate was exhaustively dialyzed against distilled water and lyophilized. The material thus obtained, in the subsequent text, has been referred as "the crude extract".

Affinity chromatography

A small column was packed with Agarose-RCA_1 and equilibrated with 50 mM Tris-HCl buffer pH 7.4. The crude extract was dissolved in a small volume of the buffer and applied on top of the column. To get rid off the unabsorbed material the column was washed by running five volumes of tris buffer. The elution was monitored by absorbance at 280 nm. It was followed by specific elution of the absorbed material with the same buffer containing 0.5 M galactose. Fractions, showing the presence of purified material were pooled, dialyzed and lyophilized.

Polyacrylamide gel electrophoresis (PAGE)

PAGE was conducted on 7% gel using tris-glycine buffer, pH 8.3 as the tank solution (Davis, 1964). The current was adjusted to the amplitude of 3 mA per tube. Protein bands were visualized by staining the gels with coomassic brilliant blue followed by destaining by several changes of methanol:acetic acid:water (5:7:88 v/v).

Determination of molecular weight

Molecular weight was determined on SDS-PAGE according to the method of Weber and Osborn (1969). Bovine serum albumin, trypsin, lysozyme and aldolase were employed as the markers. 100 ug of the test material was dissolved in 1% SDS containing 10 mM dithioerythreitol and incubated at 37°C for 1 hr. To this was added 2 mM phosphate buffer, pH 7.1 containing 8 M urea. The mixture was then exposed for 1 min to boiling water and applied on the gel.

Binding of organic solutes

In a total volume of 0.5 ml, 50-200 ug of the test material was incubated with radiolabelled compounds at 37°C. After 60 min, the reaction was stopped by the addition of an equal volume of chilled buffer. The mixture was subsequently dialyzed to remove the unbound radioactivity. Dialysates were transferred to scintillation vials and counted for radioactivity.

High performance liquid chromatography (HPLC)

Presence of various sugars in the purified preparation was detected with the aid of HPLC. For this, the purified glycoprotein was hydrolyzed at 85°C for 3 hr in presence of 2M-HCl in sealed ampoules. The hydrolysate was transferred to a small beaker and dried in a vacuum dessicator over KOH and phosphorous pentoxide. The residue was dissolved in 0.1 ml water and passed through a small Sephadex G-10 column (5 x 0.5 cm). Fractions after void volume were pooled and lyophilized. Lyophilized material was dissolved in 100-200 ul of triple distilled water. Suitable aliquot (5-10 ul) of the sample was injected to "carbohydrate analysis column" (3.9 mm width, 30 cm length) supplied with the instrument (Waters Associates Model 244) and eluted by the mixture of acetonitrile-water (85:15). Flow rate was adjusted to 0.5 ml/min and components were recorded by means of refractive detector.

Chemical nature of the individual peak was identified by comparing the retention time with standards run under identical conditions.

Circular dichroism (CD)

CD spectra of the purified glycoprotein were recorded in the range of 200-300 nm employing a Dichrograph III CNRS-Russel-Jouan Spectropolarimeter. All the measurements

were made in a quartz cell of 5 mm light path and the scan was repeated three times to get the optimum resolution. The glycoprotein was dissolved in 50 mM tris-HCl buffer, pH 7.4 to a final concentration of 33 ug/ml. Glutamic acid was added to a strength of 36 uM. The values were expressed as molar ellipticity in units of deg.cm^2/d mol, which was given by

$$\theta = 3300\ (\epsilon_L - \epsilon_R) = \frac{\theta_{obs}\ (cms) \times f \times M}{10 \times c\ 1}$$

Where, ϵ_L and ϵ_R were the extinction coefficients for left and right hand polarized light. The right hand equation is applied to experimental measurement. The curve "print-out" produced bands, provide the values for θ_{obs} (cm) in height or depth.

f = instrument sensitivity factor

M = the mean residue molecular weight (Molecular weight)

c = the concentration g/ml

l = path length in cm

θ = Molar ellipticity in degrees cm^2/decimole

α-helix content was calculated from the ellipticity at 208 nm according to the method of Greenfield and Fasman (1969).

$$f\alpha = \frac{208 + 4{,}000}{-29{,}000}$$

Assay of radioactivity

Radioactivity was measured in a Packard tricarb liquid scintillation spectrometer using 10 ml of Bray's solution (Bray, 1960). Correction for quenching was achieved using internal standard.

Protein determination

Protein in eluants was monitered by absorption at 280 nm in a Beckman DV Spectrophotometer model-24 and estimated according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Sugar determination

Neutral sugars were estimated by phenol sulphuric method described by Dubois et al. (1956). Galactose was estimated by the method of Hess and Lewin (1965).

Uptake of amino acids

To follow the transcuticular absorption, oral and anal pores of A.galli were closed by ligating the worms at both ends by two successive dippings in collodion following single predipping in acetone. Ligated worms were then incubated in KRB containing 0.1 mM ^{14}C-labelled amino acid (Specific activity 1 uCi/ umole). Incubation medium did not contain glucose since it was found to influence the absorption of amino acid. Incubation was

carried out in a Techno Shaker bath maintained at 37°C. After 2 hr parasites were removed from the medium and freed of the adhering radioactivity by thorough washing with KRB containing unlabelled amino acids. Free amino acid pool of the worms was extracted by keeping them in 70% ethanol for 2 x 12 hr. The two successive extracts were mixed together and measured for radioactivity.

Uptake of methyl glucose

Uptake of methyl glucose by S.cervi was studied in a manner similar to that described above. The worms having average weight of 25-35 mg were incubated for 30 min in KRB solution containing ^{14}C-methyl glucose (specific activity 1 uCi/ umole).

Uptake of cAMP and isoproterenol

Approximately 15 mg of adult filariids (D.viteae and L.carinii) were incubated in 2.0 ml BFM containing labelled cAMP or isoproterenol (specific activity 1 uCi/ umole).

The effect of lectins on the uptake of cAMP was studied by incubating the ligated worms in the presence of 1.0 mM cAMP containing the specified concentration of various lectins.

Analysis of carbohydrate moieties on the surface of microfilariae by flouresceinated lectins

Approximately 50,000 microfilariae of different species were incubated at 37°C with a panel of flouresceinated lectins (100 ug each) dissolved in BFM. After half an hour the tubes were centrifuged at 1,000 g for 5 minutes. The microfilarial pellet was washed by repeated suspension in BFM following centrifugation till the supernate became free of the flourescence. Subsequently the microfilarie were were suspended in 1.5 ml BFM and flourescence recorded in Spectroflourophotometer. Excitation and emission wavelengths were 490 and 520 nm respectively. Microfilariae, which were not exposed to the lectins but were treated in the similar way, provided the control values for the flourescence emitted by the parasite itself. This was substracted from the experimental values for calculating the amount of lectins bound to the parasite.

To determine the effect of drugs, microfilariae were first incubated with DEC and Centperazine (1 mM) for half an hour, washed twice through BFM and then incubated with flouresceinated lectins as described above.

CHAPTER IV

RESULTS AND DISCUSSION

SECTION A

PURIFICATION AND CHARACTERIZATION OF A GLYCOPROTEIN FROM THE CUTICULAR SURFACE OF *ASCARIDIA GALLI*

RESULTS

Data presented in Table 1 indicate that papain may be successfully employed to extract surface proteins/glycoproteins. In the present study, employing *A.galli* as the test nematode, this method provided an yield of approximately 1.5 mg of the crude extract from one gm worms. The crude extract, as it contained 5.4% carbohydrate exhibited the presence of glycoprotein(s) also. Incubation of the parasites with the enzyme for 30 min was chosen as optimum, since a contact of more than 45 minutes was found to physically damage the cuticle. Other enzymes e.g., trypsin, or pepsin did not give satisfactory results.

Table 1 - Data on crude material

Parameter	Value
Yield	1.5 mg/gm worms
Carbohydrate	5.5%
Protein	94.0%

Similarly the control set of worms incubated without enzyme did not excrete any glycoprotein in the medium. Thus for A.galli papain was found to be the most suitable enzyme.

Fig. 3 depicts the elution pattern of the crude extract during purification by affinity chromatography. It may be noticed that a large amount of the applied material left the column unabsorbed. The absorbed fraction, which was later eluted with galactose containing buffer constituted a single peak.

Electrophoretic pattern of the crude extract and the purified glycoprotein is illustrated in Fig. 4.

It may be noticed that while the crude extract exhibited the presence of approximately 14-18 types of protein bands, the purified preparation yielded a single band.

The apparent molecular weight of the isolated glycoprotein, as determined by SDS-PAGE, was found to be 68,000 daltons (Fig. 5).

Purified glycoprotein, which was found to be composed of 10% carbohydrate, when analyzed on HPLC, revealed the presence of fucose, galactose, rhamnose and glucosamine as the sugar residues (Fig. 6 and Table 2). Presence of the first two sugars was also confirmed by thin layer chromatography on silica gel employing ethyl acetate:methanol:acetic acid:water (60:15:15:10) as the solvent system (Bernard and Sherma, 1982).

Fig. 3. RCA_1 -Agarose chromatography of *A.galli* glycoprotein (crude extract) (flow rate approx. 20 ml/hr fraction volume 2 ml) (· · ·) optical density at 280 nm

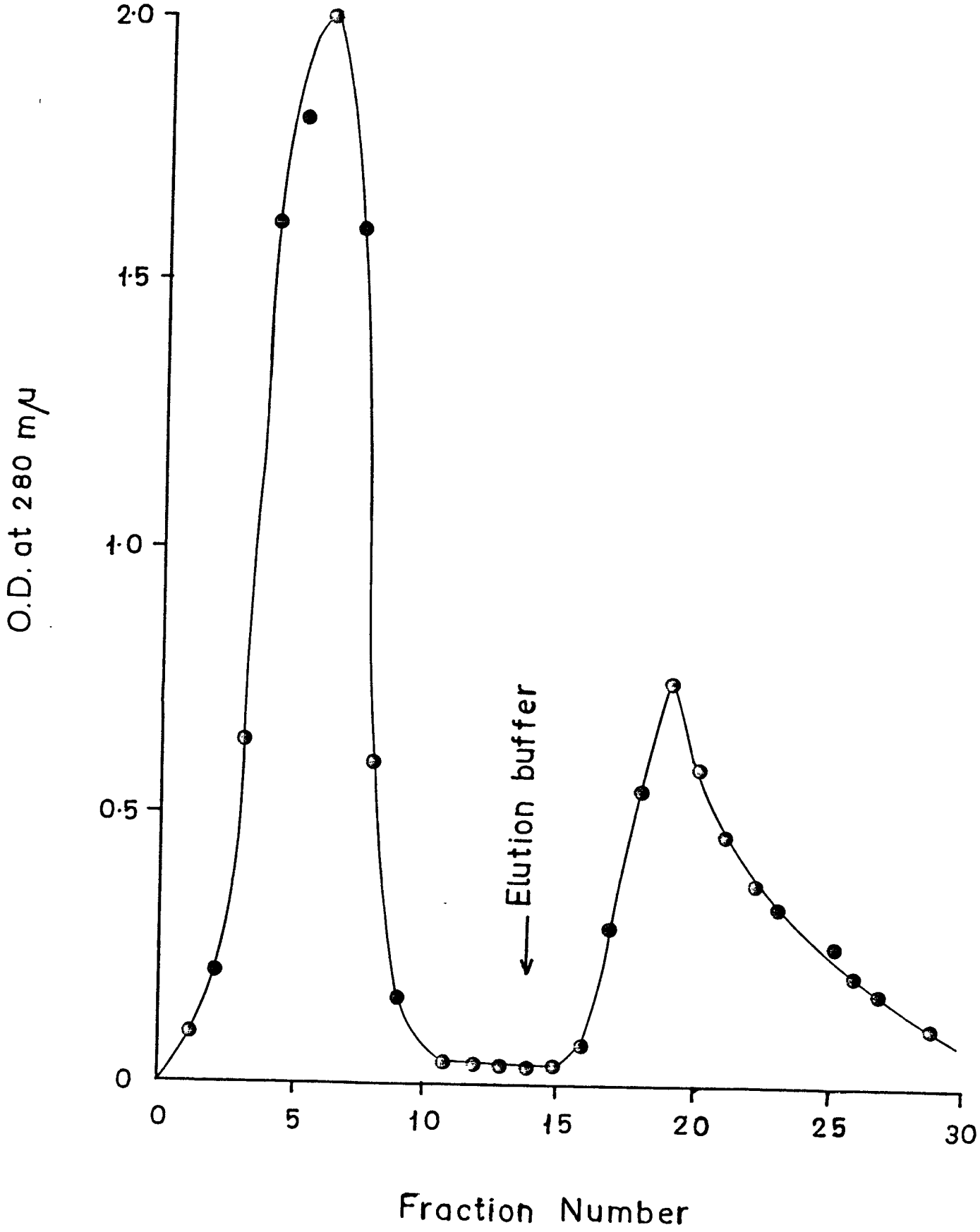

2·0
1·5
1·0
0·5
0
O.D. at 280 mμ
Elution buffer
0
5
10
15
20
25
30
Fraction Number

Fig. 4a. Polyacrylamide gel electrophoresis of crude extract.

Fig. 4b. Polyacrylamide gel electrophoresis of purified glycoprotein.

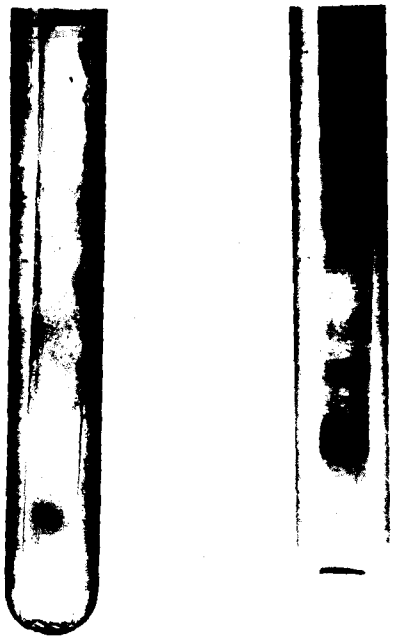


Fig. 5. Determination of molecular weight of the purified glycoprotein of *Ascaridia galli* by sodium dodecyl sulphate (SDS) gel electrophoresis. Reference proteins used were

1. Lysozyme
2. Trypsin
3. Aldolase
4. Bovine serum albumin

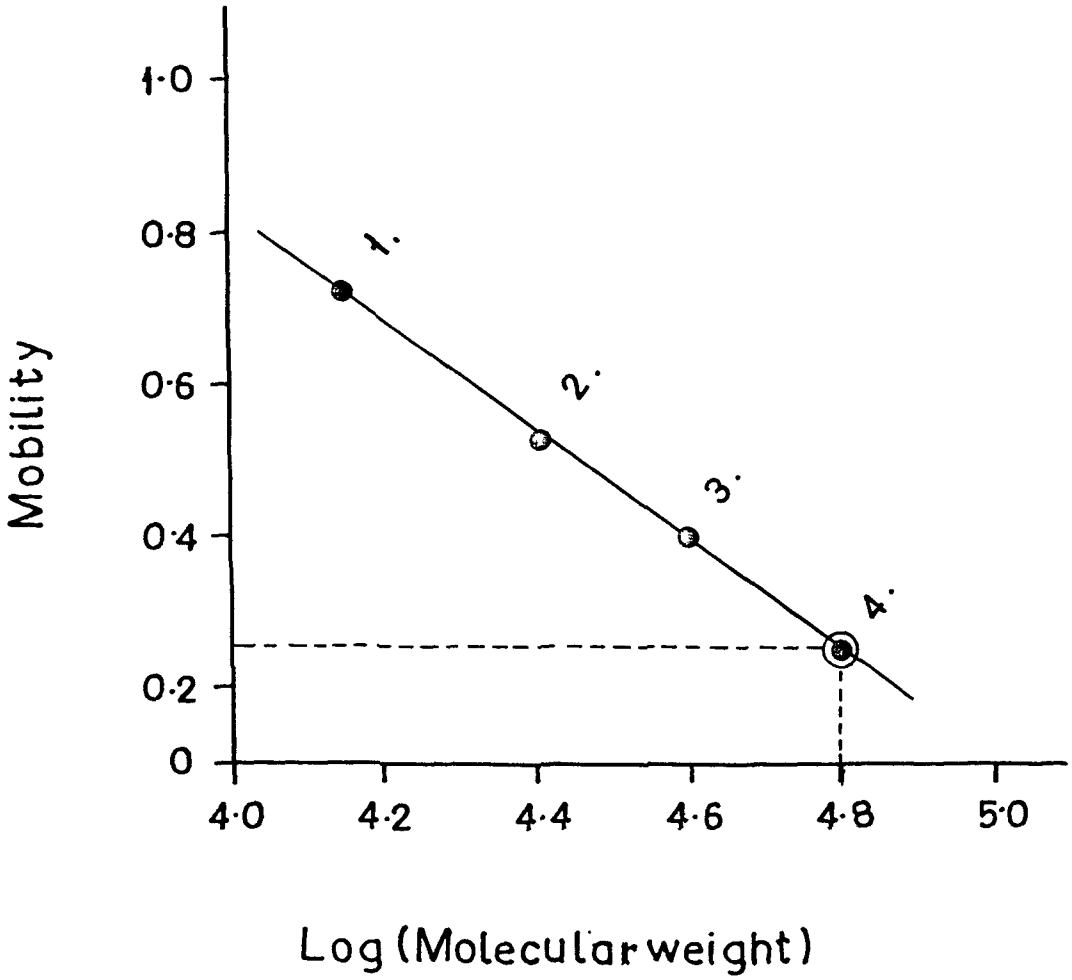

Mobility
1·0
0·8
0·6
0·4
0·2
0
1·
2·
3·
4·
4·0
4·2
4·4
4·6
4·8
5·0
Log (Molecular weight)

Table 2 - Chemical composition of the purified glycoprotein

Constituent	Value
Protein	88.5%
Carbohydrate	10.2%
Fucose	
Galactose	
Rhamnose	
Glucosamine	
Molecular weight	68,000
-Helix	2%

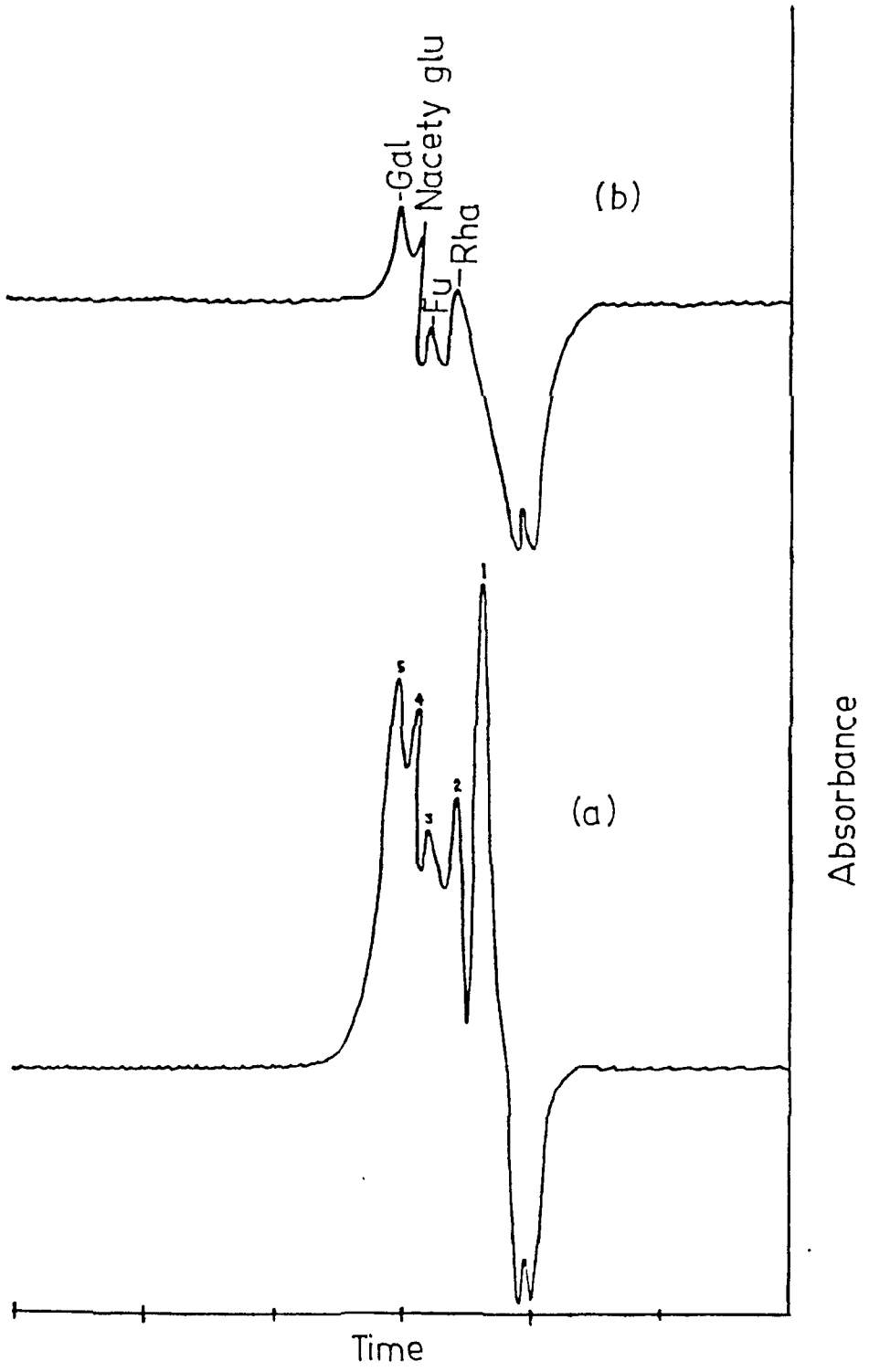

-Gal
Nacety glu
(b)
-Rha
-Fu
(a)
Absorbance
Time

Fig. 6. HPLC chromatogram of standard sugars (a) and purified protein (b).

1. Glucuronic acid = Glu acid
2. Rhamnose = Rha
3. Fucose = Fu
4. N-acetyl-glucosamine = N-acetylglu
5. Galactose = Gal

Table 3 gives an account of the comparative binding capacities of the crude and the purified glycoprotein. Out of various amino acids, serine, aspartate and glutamate showed appreciable binding with the crude extract while alanine, valine, leucine, arginine, proline, threonine did not bind to a detectable level. Among other compounds, only isoproterenol elicited good activity. Out of the above four compounds which reacted positively with the crude extract, serine, aspartate and glutamate for purified glycoprotein also exhibited significantly high affinity while isoproterenol failed to respond likewise. On quantitative basis, purification was found to result in ten and two folds increase in the binding capacity of glutamate and aspartate respectively but the affinity towards serine remain unchanged.

Ultraviolet (U.V.) absorption spectra between 200-400 nm showed the presence of a single peak at 280 nm (Fig. 7).

Fig. 8 illustrates the CD spectra of the purified glycoprotein between 200-300 nm. In the far UV region the glycoprotein (Fig. 8a) exhibited negative bands representing mainly for the polypeptide backbone. Only 2% of the entire molecule was found to have the α-helical structure. Addition of the ligand, glutamate, caused a shift of the entire spectra from negative to positive side (Fig. 8b). The helical content under this condition rose to 18%.

Table 3 - Binding property of the crude material and the purified glycoprotein

Compound	Binding activity nmoles/mg	
	Crude	Purified
Alanine	a	b
Valine	"	
Leucine	"	
Arginine	"	
Proline	"	
Threonine	"	
Serine	20.00	17.50
Aspartic acid	2.89	5.48
Glutamic acid	6.13	60.01
Glucose	a	b
Glucosamine	"	"
Glucuronic acid	"	"
cAMP	"	"
Isoproterenol	1.81	a

a = undetectable

b = not done

Values are mean of two experiments

Fig. 7. Ultraviolet absorption spectrum of the purified glycoprotein of A.galli. 40 ug glycoprotein was dissolved in 50 mM Tris-HCl buffer pH 7.4.

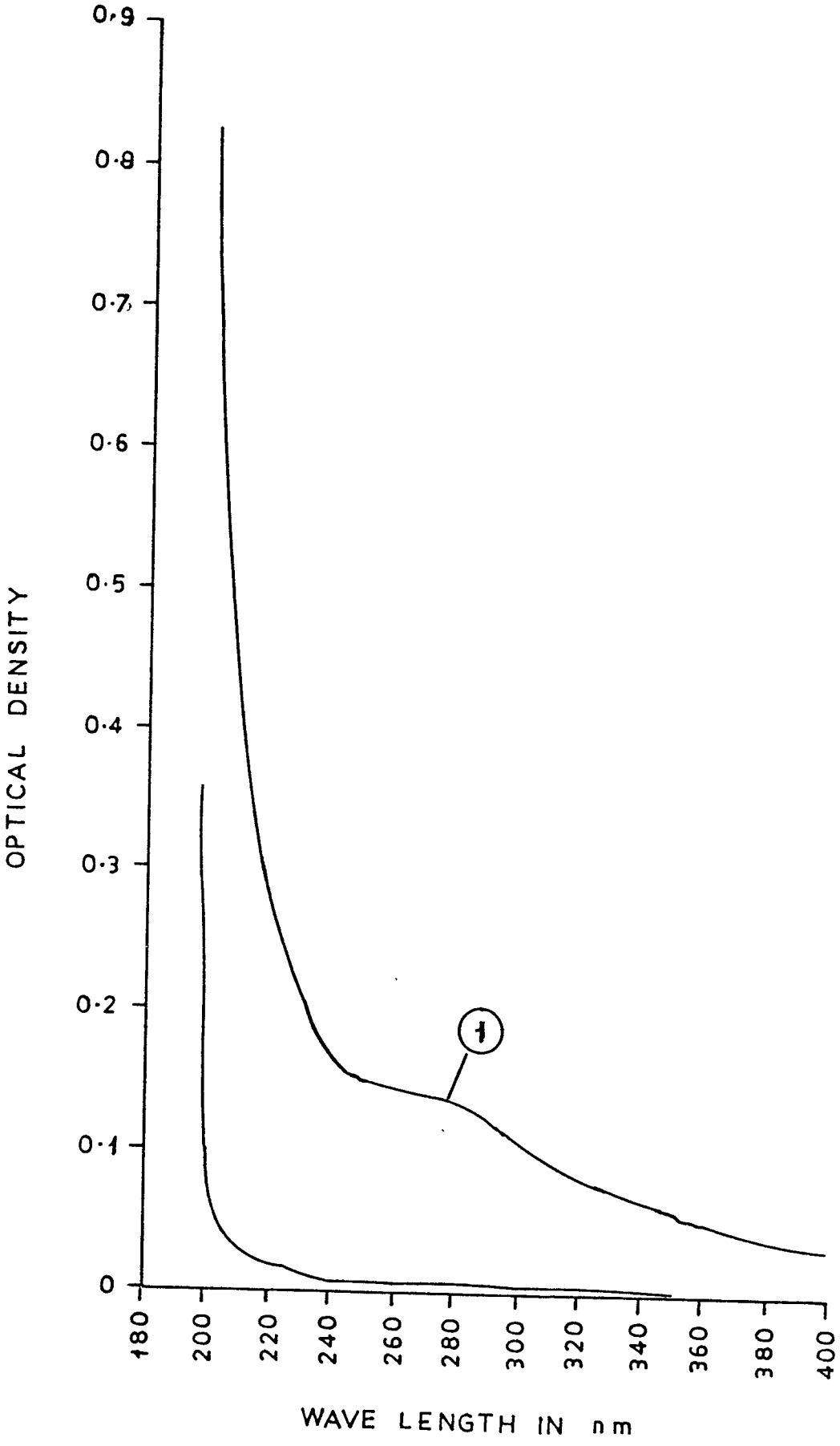
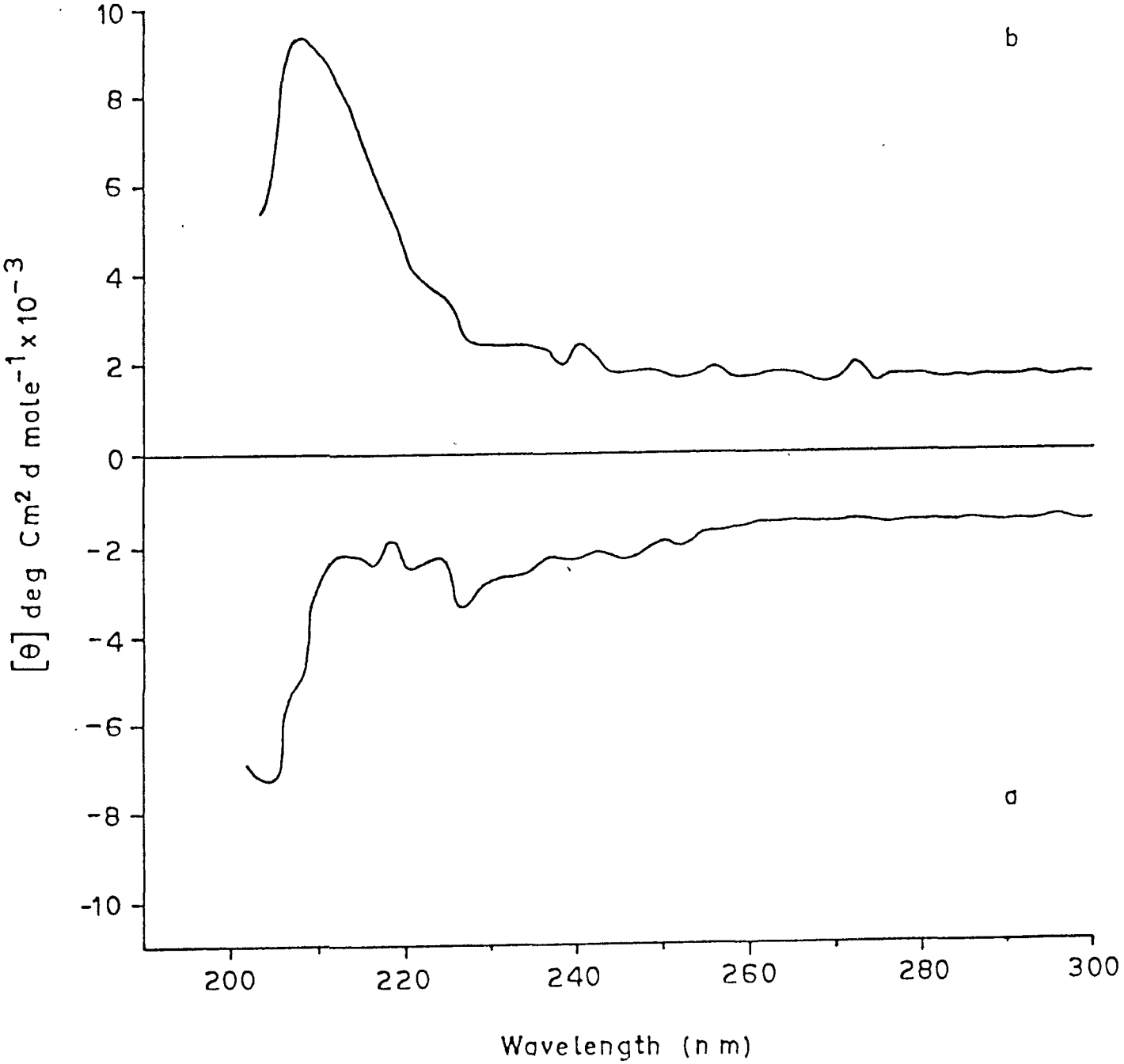

0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
OPTICAL DENSITY
1
180
200
220
240
260
280
300
320
340
360
380
400
WAVE LENGTH IN nm

Fig. 8. CD spectra of the purified glycoprotein (a) and its complex with glutamic acid (b).


10
8
6
4
2
0
-2
-4
-6
-8
-10
[θ] deg Cm² d mole⁻¹ x 10⁻³
200
220
240
260
280
300
Wavelength (n m)
b
a

DISCUSSION

Chemical constituents of the parasites surface, particularly the proteins and glycoproteins are thought to be involved in a number of versatile functions, such as the transport of metabolites (Pappas and Read, 1975), antigenic response (Cross, 1975) and interactions with the exogenous and endogenous types of substances present in the environment (Beldekas and Pissens, 1979; Buys *et al.*, 1981; Srivastava *et al.*, 1984; Subrahmanyam *et al.*, 1978; Tanner and Weiss, 1978). Though a number of agents for the isolation of these entities from mammalian sources have been described in literature (Glick, 1974), trypsin has been successfully employed with a few parasites (Rosen *et al.*, 1981; Dissous *et al.*, 1981). The failure of trypsin to isolate the surface substances, in the present study may be attributed to the inactivation of the enzyme by its inhibitor shown to be possessed (Ansari *et al.*, 1976) and released into the environment (Srivastava, VML unpublished) by *A.galli*. Interestingly employing papain as the chopping agent, appreciably good quantity of the crude extract (1.5 mg/g worm, Table 1), possessing number of proteins/glycoproteins (Fig. 4) has been isolated from the surface of this nematode. This indicates that papain may be a better extracting agent, as the chances for the presence of its inhibitor in

animal or helminth systems are very remote. Affinity of the crude extract towards serine, aspartic acid, glutamic acid and isoproterenol (Table 3) clearly indicates that the surface of A.galli is equipped with the system(s) for the interaction of these molecules. This system may be involved in the transport or metabolic regulation. Purification of a single glycoprotein (Fig. 4) from the crude by chromatography on Agarose-RCA_1 (Fig. 3) provides a strong support for the use of affinity chromatography with surface molecules also. The clue to select the affinity gel has been obtained by the detection of galactose in the crude extract. The purified glycoprotein as usual has been found to contain fucose, galactose, rhamnose and glucosamine as the sugar moieties Table 2). Interestingly, the serologically active protein polysaccharide complex from Trichomonas foetus is also reported to contain rhamnose, fucose, galactose, xylose and hexosamine (Alves and Colli, 1975). A similar complex isolated from Trypanosoma cruzi contains glucosamine, galactose, glucose, mannose and xylose in its polysaccharide moiety, which appears to be responsible for the immunological property of the complex (Goncalves and Yamaha, 1969).

Since about a ten and two folds increase in the binding capacity for glutamate and aspartate respectively has been illustrated by the purified glycoprotein over

the crude extract (Table 3), it indicates a specific interaction between the two molecules. This view gets support from the CD spectra (Fig. 8) also, as the fixation of the ligand brings about a complete shift of the spectrum from negative to positive side. The binding also causes conformational changes which is reflected by the increase in helical content of the molecule from 2 to 18%. Fig. 7 showing the presence of a single peak at 280 nm indicates towards the normal absorption behaviour of pure protein.

Negative binding, as observed with glucose, cAMP and other amino acids, does not necessarily indicates that the systems for their interaction are not present on the cuticular surface of A.galli. It nevertheless indicates that such constituents, if any present, may not be isolated by the procedure employed in this study. Glycoproteins which bind with serine, aspartate and glutamate may be involved in the transcuticular absorption of these compounds. The two amino acids have been found to be absorbed through the cuticle (see Table 7). Binding with isoprotenenol is of particular interest, since the compound in mammalian system is known to participate in regulation through cAMP (Jost and Rickenberg, 1971). Though the regulatory role of this compound in helminths has not been studied so far, cAMP is

known to affect the metabolism in a few parasites including *Ascaris lumbricoides* (Donahue *et al.*, 1981) and *A.galli* (Roy and Srivastava, 1981). It therefore, appears that *A.galli* possesses cuticular system(s) through which it can adapt to the environmental stresses.

SECTION B

UPTAKE THROUGH THE CUTICLE OF NEMATODE PARASITES

In spite of the presence of a well developed digestive system in nematodes, the involvement of their cuticle in absorption is an important aspect of the uptake of nutrients. Earlier studies have shown the *in vivo* and *in vitro* incorporation of labelled glucose and amino acids in the body of the parasite (Hankes and Stoner, 1956; Hankes and Stoner, 1958; Stoner and Hankes, 1955; Stoner and Hankes, 1958), but no attempts have been made to identify the route of their incorporation. Though a few reports indicating transcuticular movement (Pavlov, 1976; Pavlov and Volynskaya, 1970; Pavlov and Koshkina, 1974; Drynchenko and Shishov, 1974), but the general view is that the intestine is probably the major route of absorption of nutrients.

In this chapter results on the uptake of a number of compounds by A.galli as well as by filarial parasites have been discussed in details. Attempts have also been made to identify of nature of the existing mechanisms and the uptake of various other substances on these process.

PART 1: TRANSCUTICULAR ABSORPTION OF AMINO ACIDS BY ASCARIDIA GALLI

In this section the effect of age and sex as well as the mechanism of uptake of amino acids have been described.

RESULTS

Since both the anal and oral openings of A.galli have been ligated, the uptake by such parasites represents transcuticular absorption. Data summarized in Table 4 indicate that unligated worms compared to ligated ones, in general accumulated higher amounts of amino acids.

Sex does not appear to affect the uptake to a considerable extent except of having a slight difference at the quantitative level since the parasites of both the sexes displayed similar pattern (Table 5). Thus, both male and female worms absorbed proline and lysine at highest rate. Serine, leucine and aspartic acid were taken up at moderate rates, while alanine and arginine showed minimum absorption.

Table 4 - In vitro uptake of amino acids in Ascaridia galli*

Amino acids	Mature worms	
	Unligated	Ligated
Alanine	1.3106	0.931
Arginine	1.089	0.908
Aspartic acid	2.890	2.540
Glycine	1.562	1.321
Glutamic acid	2.310	1.980
Leucine	4.492	2.521
Lysine	4.621	4.305
Methionine	0.625	1.411
Proline	6.462	4.960
Serine	4.056	3.592
Tyrosine	2.361	1.749
Valine	2.718	1.688

* nmoles/g worms/hr

Values are mean of two experiments

Table 5 - *In vitro* uptake of amino acids in *A.galli*[*]

Amino acids	Mature worms	
	Ligated female	Ligated male
Alanine	0.931	0.878
Arginine	0.908	0.772
Aspartic acid	2.540	2.210
Glycine	1.321	1.031
Glutamic acid	1.980	2.051
Leucine	2.521	2.411
Lysine	4.305	4.165
Methionine	1.411	1.144
Proline	4.960	4.560
Serine	3.592	3.650
Tyrosine	1.749	1.499
Valine	1.688	1.348

* nmoles/g worms/hr

Values are mean of two experiments

Age of the parasite however, was found to have a great influence; younger parasites utilized these acids at relatively faster rate than the older ones (Table 6). Quantitatively, the uptake by young A.galli ranged between 1.5 to 3.0 folds over that by the mature worms. Nonetheless, the relative uptake of amino acids remained unchanged. For example, the order for uptake of proline, serine and lysine etc. was the same for either group of worms.

Uptake was found to be a concentration dependant process. Thus, proline and serine exhibited a parallel increase in uptake with respect to their increasing concentration in the ambient medium (Table 7). When analyzed by coordinate geometry, the values held a linear relationship and did not show saturation even at the highest concentration used (Fig. 9). This indicated that these amino acids crossed the cuticle by simple diffusion.

DISCUSSION

Though a great controversy occurs regarding the permeability of nematode cuticle for low molecular weight polar solutes (Pappas and Read, 1975), present investigation clearly indicates that in A.galli transcuticular absorption of amino acids does take place. Weatherly et al. (1963) have also demonstrated the uptake of alanine

Table 6 - *In vitro* uptake of amino acids in *A.galli*[*]

Amino acids	Young worms	
	Unligated	Ligated
Alanine	2.894	2.456
Arginine	3.672	2.883
Aspartic acid	2.920	2.996
Glycine	3.224	2.951
Glutamic acid	4.738	3.126
Leucine	1.913	1.603
Lysine	5.011	5.488
Methionine	1.234	2.142
Proline	8.778	6.807
Serine	8.121	6.130
Tyrosine	3.807	3.127
Valine	5.545	4.327

* nmoles/g worms/hr

Values are mean of two experiments

Table 7 - Uptake of proline and serine in adult *A.galli**

Concentration (mM)	Rate of uptake	
	Proline	Serine
0.01	1.2	0.005
0.02	1.7	0.893
0.05	3.3	1.93
0.1	4.8	3.84
0.2	8.5	4.00
0.5	29.4	9.54
1.0	46.2	17.83

* nmoles/g worms/hr

Values are mean of two experiments

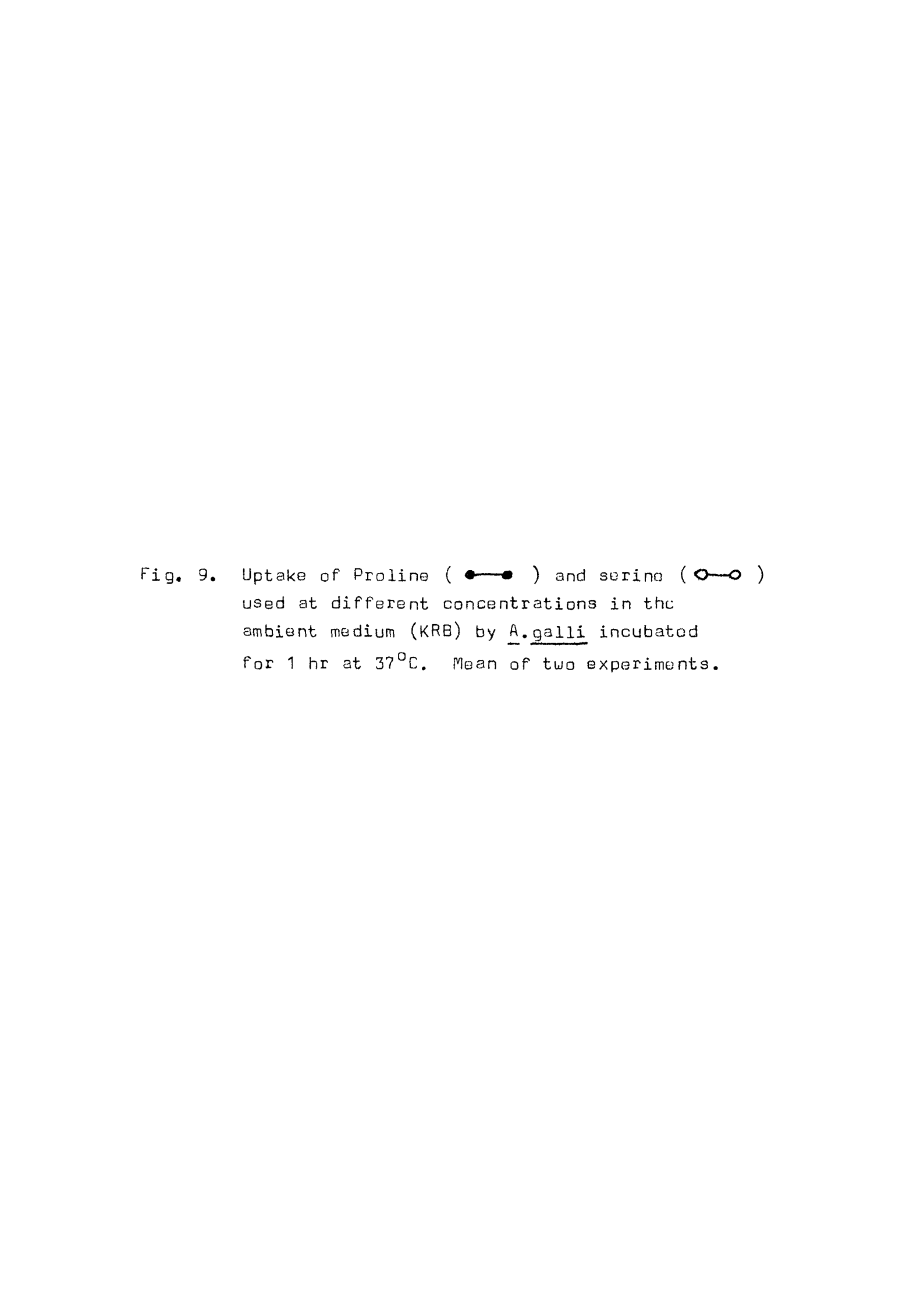
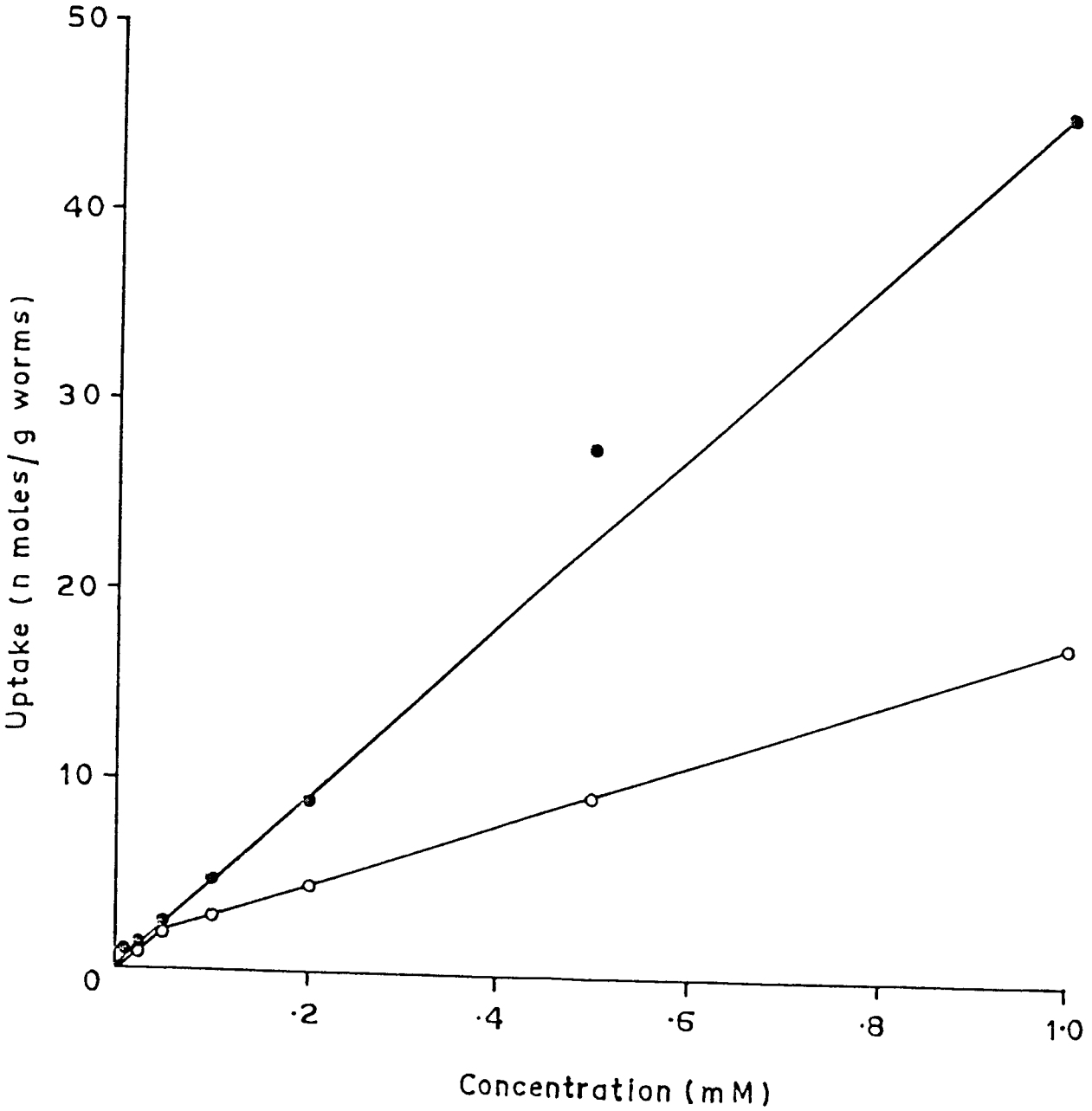


Fig. 9. Uptake of Proline (●—●) and serine (○—○) used at different concentrations in the ambient medium (KRB) by A.galli incubated for 1 hr at 37°C. Mean of two experiments.


50
40
30
20
10
0
Uptake (n moles/g worms)
·2
·4
·6
·8
1·0
Concentration (mM)

and glucose to occur through the cuticle of this nematode. Furthermore, electron microscopic detection of structures resembling microvilli in nematode cuticle (Lumsden, 1975) also suggests towards the absorptive role of the cuticular surface. Interestingly during the last four-five years strong evidences in support of the movement of various types of compounds through nematode cuticle have been presented by a number of workers (Chapter II). The most interesting among them to mention are the autoradiographic studies of Chen and Howells (1979) and Howells and Chen (1981) with Brugia pahangi. They have presented evidence regarding transcuticular absorption of sugars, amino acids, nucleotides and anthelmintics by the filarial parasite Brugia pahangi.

Hence the increasing number of reports are in favour of absorptive role of the nematode cuticle and results of the present investigation also supports this concept.

PART 2: TRANSCUTICULAR UPTAKE OF METHYL GLUCOSE BY SETARIA CERVI

In this section the mechanism by which methyl glucose is transported across the cuticle of Setaria cervi has been identified. Attempts have also been made to correlate the effects of certain agents on the motility of the worms and on the uptake of the sugar.

RESULTS

The uptake of methyl glucose by S.cervi was found to take place as a function of concentration by ingestion as well as by transcuticular absorption (Table 8). The uptake observed in unligated worms, in comparison to the ligated parasites, was higher at each concentration used. Such an uptake was biphasic in nature, since upto 2 mM concentration the ratio of uptake in the unligated and ligated worms gradually decreased whereas above 2 mM concentration this ratio increased to 2.15.

Fig. 10 was a velocity concentration curve for the uptake of methyl glucose by the ligated parasite. The curve was a hyperbolic with saturation around 6 mM of substrate methyl glucose. These results thus indicated that transcuticular movement of methyl glucose could presumably be accomplished by a carrier dependent system. A double reciprocal plot (Lineweaver and Burk, 1934) appeared as the inset in Fig. 10. This plot exhibited a value of 1.67 mM as the transport constant (K_t) and of 3.57 u mole/g/hr as V_{max} for the uptake of methyl glucose.

As is evident from Fig. 11, the accumulation of ^{14}C-methyl glucose into the free pool component (extractable with 70% ethanol) of nematode against time showed a lag period upto 30-45 min, after which the rate of uptake

Table 8 - Uptake of methyl glucose by S.cervi

Concentration (mM)	Uptake (umole/g/hr)		Ratio*
	Unligated	Ligated	
0.1	0.40	0.20	2.00
0.5	1.40	0.80	1.62
1.0	2.50	1.50	1.50
2.0	3.70	2.60	1.42
4.0	4.70	3.10	1.45
6.0	5.50	3.20	1.72
10.0	6.00	3.25	1.84
15.0	6.50	3.30	1.97
20.0	7.00	3.30	2.12
40.0	7.00	3.25	2.15

* Uptake by unligated/uptake by ligated worm.

Fig. 10. Uptake of methylglucose by S.cervi as a function of sugar concentration. Curve in the inset represents double reciprocal plot.

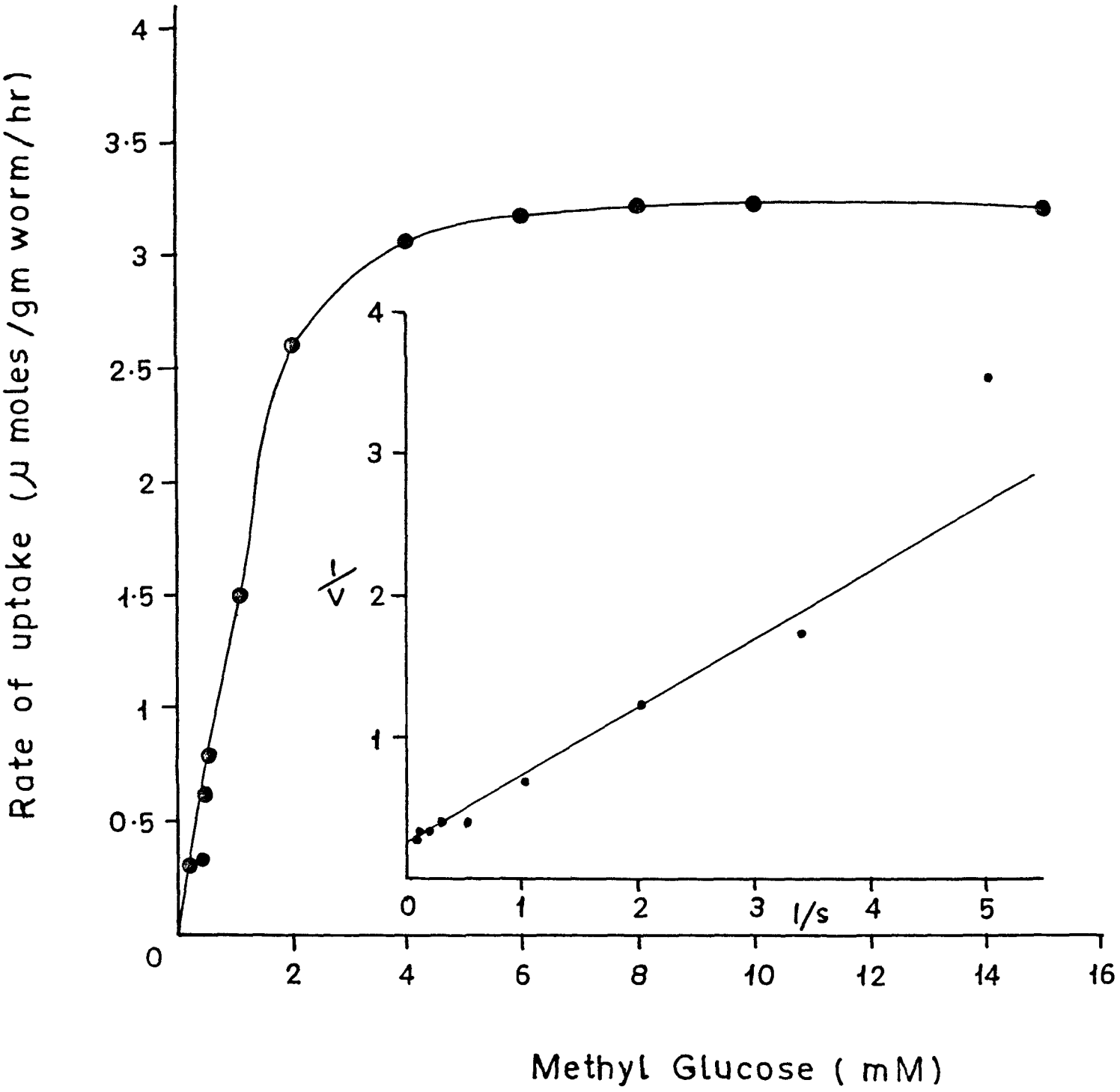
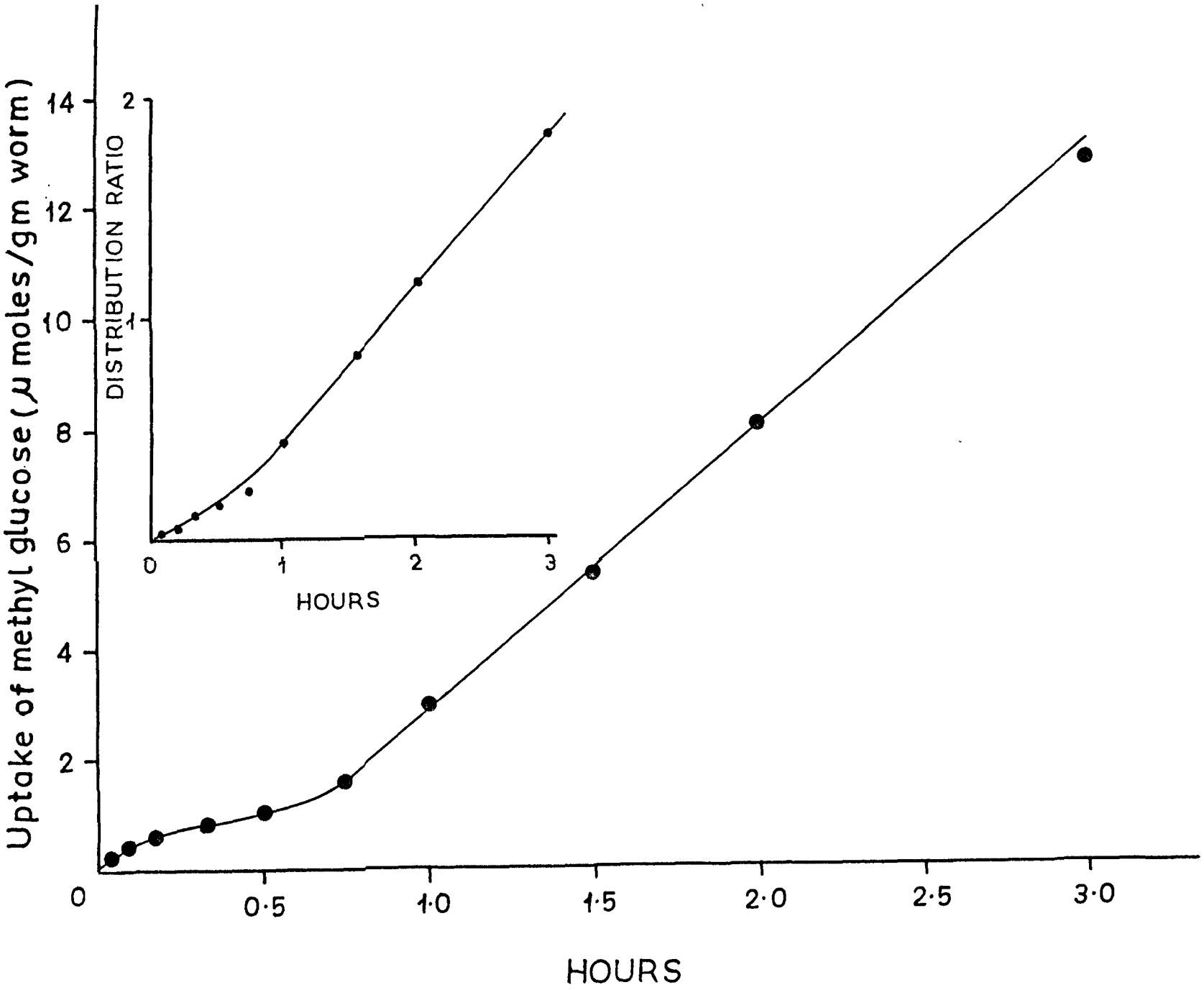

Rate of uptake (μ moles/gm worm/hr)
4
3·5
3
2·5
2
1·5
1
0·5
0
2
4
6
8
10
12
14
16
Methyl Glucose (mM)
1/V
4
3
2
1
0
1
2
3
1/s
4
5

Fig. 11. Uptake of methylglucose by S.cervi as a function of time. Figure in the inset illustrates the relationship between the distribution ratio and the time of incubation.


Uptake of methyl glucose (μ moles/gm worm)
14
12
10
8
6
4
2
0
0·5
1·0
1·5
2·0
2·5
3·0
HOURS
DISTRIBUTION RATIO
2
1
0
1
2
3
HOURS

suddenly increased and remained constant upto the study period of 3 hr. The curve in the inset (Fig. 11) represents the distribution ratio of radioactivity between tissue water and the medium. The ratio of distribution is as high as 2.4. Water content of fresh worms (76.7%) has been taken as the tissue water. If the difference between the wet weight and the ethanol-extracted dry weight of the parasite, first used by Read *et al.* (1963), is assumed to be the worm water value (82.4% for *S.cervi*), then the highest distribution ratio of methyl glucose between the worm water and the medium by this factor will be 1.86, indicating the existence of a possible active transport mechanism in *S.cervi*.

Phloridzin, a specific inhibitor of glucose transport, was found to inhibit the sugar uptake in *S.cervi* (Fig. 12). The inhibition increased sharply upto 10^{-6}M, after which no further increase was observed. The 50% inhibitory effects were caused by 10^{-4}M concentration of the inhibitor. The uptake of methyl glucose by the filarial parasite was also found to be inhibited by triphenyltin chloride and pHMB. These agents, in contrast, to phloridzin, showed a linear pattern of inhibition against the log of their molar concentration. The effects of the above three inhibitors on the motility of *S.cervi* are summarized in Table 9. It may be noticed

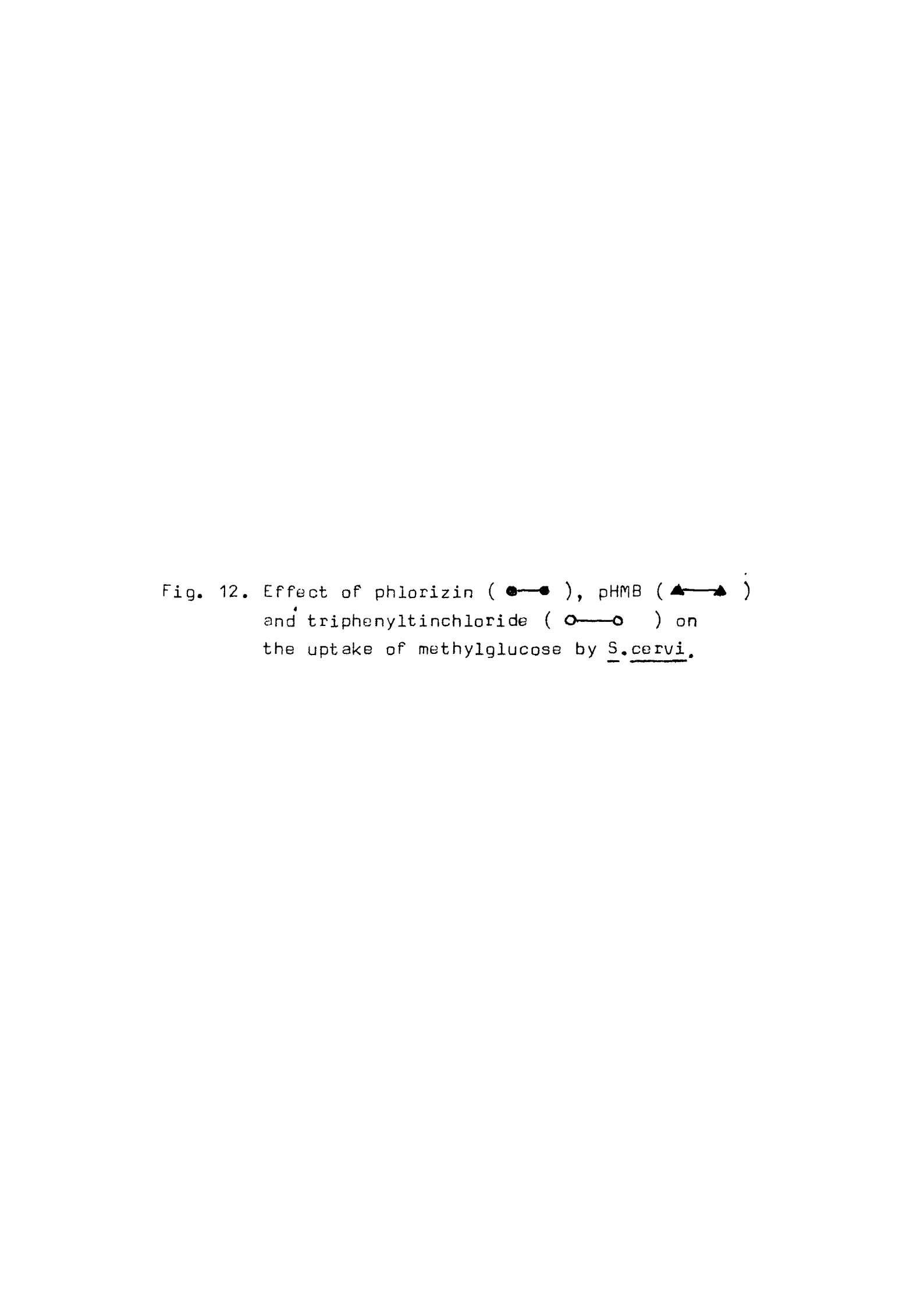


Fig. 12. Effect of phlorizin (●—●), pHMB (▲—▲) and triphenyltinchloride (o—o) on the uptake of methylglucose by S.cervi.

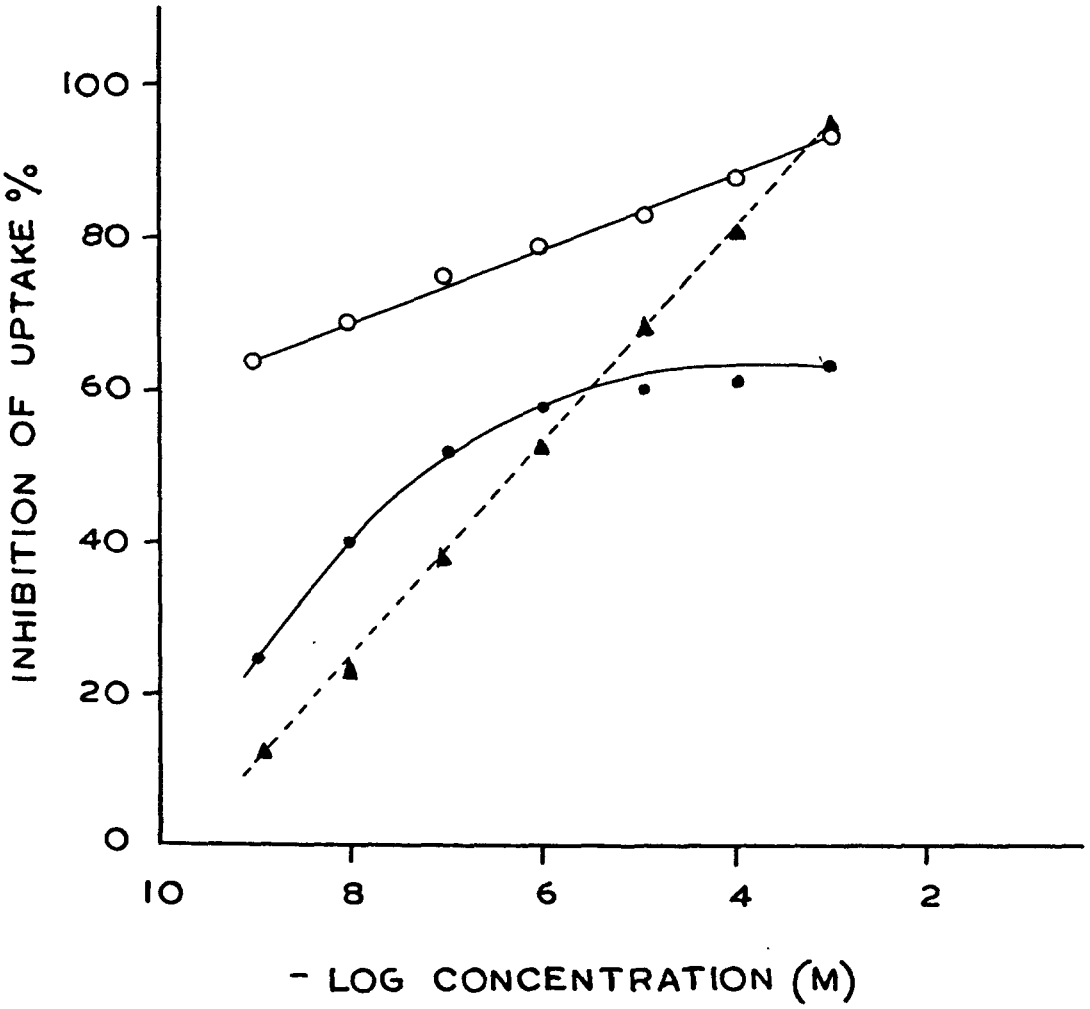

INHIBITION OF UPTAKE %
100
80
60
40
20
0
10
8
6
4
2
- LOG CONCENTRATION (M)

Table 9 - Effect of triphenyltin chloride, pHMB and phlorizin on motility of *S.cervi*

Inhibitor conc. (M)	Worm activity*					
	Triphenyltin chloride		pHMB		Phlorizin	
	A	B	A	B	A	B
None	+++	+++	+++	+++	+++	+++
1×10^{-3}	0	0	++	-	+++	+++
5×10^{-4}	0	0	++	-	+++	+++
1×10^{-4}	++	-	+++	+	+++	+++
5×10^{-5}	++	-	+++	+	+++	+++
1×10^{-5}	+++	+	+++	++	+++	+++
5×10^{-6}	0	0	+++	+++	+++	+++
1×10^{-6}	+++	++	+++	+++	+++	+++
5×10^{-7}	+++	++	+++	+++	+++	+++
1×10^{-7}	+++	+++	+++	+++	+++	+++
5×10^{-8}	+++	+++	+++	+++	+++	+++

* +++, active movement; ++, moderate movement; +, sluggish movement; -, no movement; 0, not studied.
A, 30 min.incubation and B, 60 min.incubation.

that triphenyltin chloride and pHMB at higher concentrations reduced the mobility of the worm within 30 min and apparently killed them in one hour. Phloridzin, however, did not show any apparent effect on the mobility of these worms. The effects of triphenyltin chloride and pHMB appeared to be irreversible in nature as evidenced by the observations that preincubation of the worms with these inhibitors was unable to restore the sugar uptake control values (Table 10). Effect of phloridzin, on the other hand, was found to be reversible since incubation of worms after inhibition in fresh inhibitor free medium recovered the uptake.

Transcuticular uptake of methyl glucose by the parasite was also inhibited by 2,4-dinitrophenyl (DNP) and potassium cyanide (KCN) (Table 11). The inhibitory effects of DNP were, however, greater since 10^{-3}M DNP caused 30% reduction of sugar uptake as compared to only 10% observed with KCN. Both DNP and KCN also like phloridzin did not exhibit any effects on the motility of these worms.

Preincubation of the worms in the presence of glucose, mannose and ribose reduced the uptake of methyl glucose by S.cervi, whereas similar preincubation in medium containing galactose and fructose increased the absorption rate (Table 12).

Table 10 - Effect of post-inhibitor-free incubation on uptake of methylglucose by S.cervi preincubated with triphenyltin chloride, pHMB and phlorizin*

Inhibitor	Concentration (M)	Uptake (umole/g/hr)	Uptake (%)
None	-	3.25	-
Triphenyltin chloride	1×10^{-5}	0.39	12.0
	1×10^{-6}	0.52	16.0
	1×10^{-7}	0.68	21.0
pHMB	1×10^{-5}	0.81	25.0
	1×10^{-6}	1.22	37.5
	1×10^{-7}	1.50	46.2
Phlorizin	1×10^{-3}	3.20	98.5
	1×10^{-4}	3.30	101.5
	1×10^{-5}	3.41	104.9

* Worms were preincubated with inhibitors for 30 min, washed well with inhibitor-free incubating medium and postincubated in inhibitor-free medium containing 10 mM methylglucose for 30 min.

Table 11 - Effect of DNP and KCN on transcuticular uptake of methylglucose

Inhibitor	Concentration (M)	Uptake (umole/g/hr)	Inhibition (%)
None	0	3.15	-
DNP	10^{-6}	3.15	0
	10^{-5}	2.84	10
	10^{-4}	2.20	30
	10^{-3}	1.01	68
	10^{-2}	0.47	85
KCN	10^{-5}	3.15	0
	10^{-4}	2.85	10
	10^{-3}	2.36	25
	10^{-2}	1.89	40

Table 12 - Effect of preincubation with various monosaccharides on uptake of methylglucose*

Monosaccharide	Uptake (umole/g/hr)	Uptake (%)
None	3.20	-
Glucose	2.42	75.7
Mannose	2.67	83.3
Ribose	2.79	87.3
Fructose	4.55	142.3
Galactose	3.75	117.1

* Worms were preincubated for 15 min in 10 mM solution of each monosaccharide and then incubated for 30 min with 10 mM methylglucose.

DISCUSSION

The results indicate that the uptake of methyl glucose in S.cervi occurs through ingestion as well as through the transcuticular absorption (Table 8). Functioning of the latter mechanism has been clearly demonstrated by blocking the ingestion through mouth by ligating the worms with collodion. Under this condition, the absorption of sugar occurs solely by active transport (Fig. 11) at a maximum rate of 3.57 umoles/gm/hr. Diffusion does not appear to play a role in the absorption of this sugar (Fig. 10). Uptake of glucose through active transport has also been suggested to occur in mermithid nematode, Mermis nigrescens (Rutherford and Webster, 1977). In addition to the above, pores through which ferritin particles could traverse, have recently been shown in the cuticle of another mermithid nematode, Romanomermis culcivorax (Poinar and Hess, 1977). Results of the present study are in agreement with those of Chen and Howells (1979) and these observations clearly indicate that the movement of small molecules through cuticle is not limited to intestinal nematodes only (Weatherly et al., 1963; Harris et al., 1972) but it occurs in filarial parasites also.

The maximum inhibition of sugar uptake by 63% in the presence of 10^{-3}M phloridzin (Fig. 12) which acts as a competitive inhibitor, indicates that the concentration of phloridzin used was too low in respect to the concentration of the substrate (10 mM) to cause complete inhibition. However, the concentrations above 10^{-3}M could not be used due to their ability to cause mitochondrial swelling (Crane, 1960).

Triphenyltin chloride caused a concentration dependent irreversible inhibition of the sugar transport in S.cervi (Fig. 12) and apparently killed the parasite (Table 9). Though the effect of this tin-compound on the metabolism of this parasite has not been studied, all the observations discussed above strongly suggest that triphenyl tin chloride possibly acts like other tin compounds (Aldridge and Cremer, 1955; Mitchell, 1963; Stockdale et al., 1970) by impairing the energy generating system of the parasite or by binding at the transport site.

pHMB, which mimiced triphenyltin chloride in all respects (Fig. 12, Tables 9 and 10), also appears to act by interfering either with the energy metabolism or by binding to the transport site. Hg^{2+} is known to inhibit glucose uptake by rat diaphragm as well as the respiration (Demis and Rothstein, 1955).

DNP and KCN both inhibited the transcuticular absorption of methyl glucose by S.cervi (Table 11). DNP, however, exhibited more pronounced inhibitory effects. The results obtained with S.cervi are not surprizing, since KCN at 1×10^{-3}M does not affect mitochondrial production of ATP while DNP strongly inhibits the energy output by this filarial worm (Singh et al., 1984).

Preincubation of the worms in the presence of metabolizing sugars, viz. glucose, mannose and ribose has been found to lower the net uptake of methyl glucose. Preloading with the nonmetabolizable sugars like fructose and galactose (Anwar et al., 1975), on the contrary, increase the influx of methyl glucose (Table 12). This indicates that the nature of the sugar with respect to its energy yielding capability, has significant effect on their capacity to influence the rate of entry of methyl glucose through the cuticle of the parasite.

It may finally be concluded that in S.cervi a physiologically significant amount of sugar, in addition to ingestion, is taken up by transcuticular absorption. The process involved in the movement of the sugar appears to be an active transport, where metabolic inhibitors of energy production cause reversible or irreversible effects depending on their nature.

PART 3: UPTAKE OF ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE AND ISOPROTERENOL BY FILARIAL PARASITES

This section deals with the comparative uptake of two metabolic regulators, cAMP and isoproterenol by two filarial parasites, L.carinii and D.viteae. The effect of various lectins has been investigated with a view to describe the role of various sugar moieties found at the surface of the parasite in the uptake processes.

RESULTS

The observed uptake of cAMP by the two filariids is summarized in Table 13. Both parasites absorb the nucleotide through their cuticle, no significant differences between the uptake values of the ligated and unligated worms being recorded. Though L.carinii absorbed comparatively less nucleotide than did D.viteae, uptake by both increased with increasing solute concentration in the ambient medium.

The pattern of uptake of isoproterenol (Table 14) differs markedly from that of cAMP. It is interesting that L.carinii does not absorb the compound to a detectable level whereas D.viteae does.

Table 15 shows the effect of various lectins on the in vitro uptake of cAMP by L.carinii and D.viteae.

Table 13 - Uptake of cAMP by filarial parasites*

Concentration (mM)	D.viteae		L.carinii
	Unligated	Ligated	Ligated
0.1	1.6	1.3	0.06
0.4	3.5	3.8	0.17
1.0	6.6	6.1	0.46

* u moles/gm worms/hr

Values are mean of two experiments

Table 14 - Uptake of Isoproterenol by filarial parasites*

Concentration (mM)	D.viteae	L.carinii
1.0	1.3	Un=
1.5	4.2	Un
2.0	5.2	Un

* u moles/gm worms/hr by ligated worms

= undetectable

Values are mean of two experiments

Table 15 - Effect of lectins on the uptake of cAMP by filarial parasites*

Additions	Concentration (mg/ml)	D.viteae	L.carinii
None	-	6.00	0.48
Con-A	1.0	3.12	0.28
RCA_1	0.1	2.38	0.24
RCA_2	0.1	1.75	0.16
PNA	0.025	5.86	-
	0.100	2.90	0.17
	0.200	1.80	-

* umoles/gm worms/hr

Values are mean of two experiments

All the lectins, concanavalin-A (Con-A), Ricinus cummunis agglutinin (RCA_1), ricin (RCA_2) and peanut agglutinin (PNA), which bind to various sugar ligands, strongly inhibit uptake of the cyclic nucleotide. This inhibition is dependent on the concentration of PNA, as an increase in PNA concentration produces a parallel inhibition of uptake.

Fig. 13 is a velocity concentration curve for uptake of cAMP by D.viteae. The curve is a hyperbolic with saturation around 5 uM of cAMP. This indicates that uptake of cAMP takes place through active transport with significant component of diffusion. The area between solid line and dotted line indicates the diffusion process. A double reciprocal plot (Lineweaver and Burk, 1934) is presented in Fig. 14. The transport constant (K_t) of cAMP is 0.15 mM and the maximum velocity (V_{max}) of transport is 0.672 umol/gm/min.

Fig. 14 indicates the accumulation of ^3H-cAMP into the pool component of D.viteae against time. The rate of uptake is constantly increasing upto the study period of 90 minutes. The curve in the inset (Fig. 15) represents the relationship between time and the distribution ratio of radioactivity. The ratio of distribution is as high as 8.1. Water content of fresh worms (70%) has been taken as the tissue water. The distribution ratio 8.1 of cAMP between D.viteae water and the medium indicated the existence of active transport mechanism.

Fig. 13. Uptake of cAMP by D.viteae as a function of cAMP concentration.

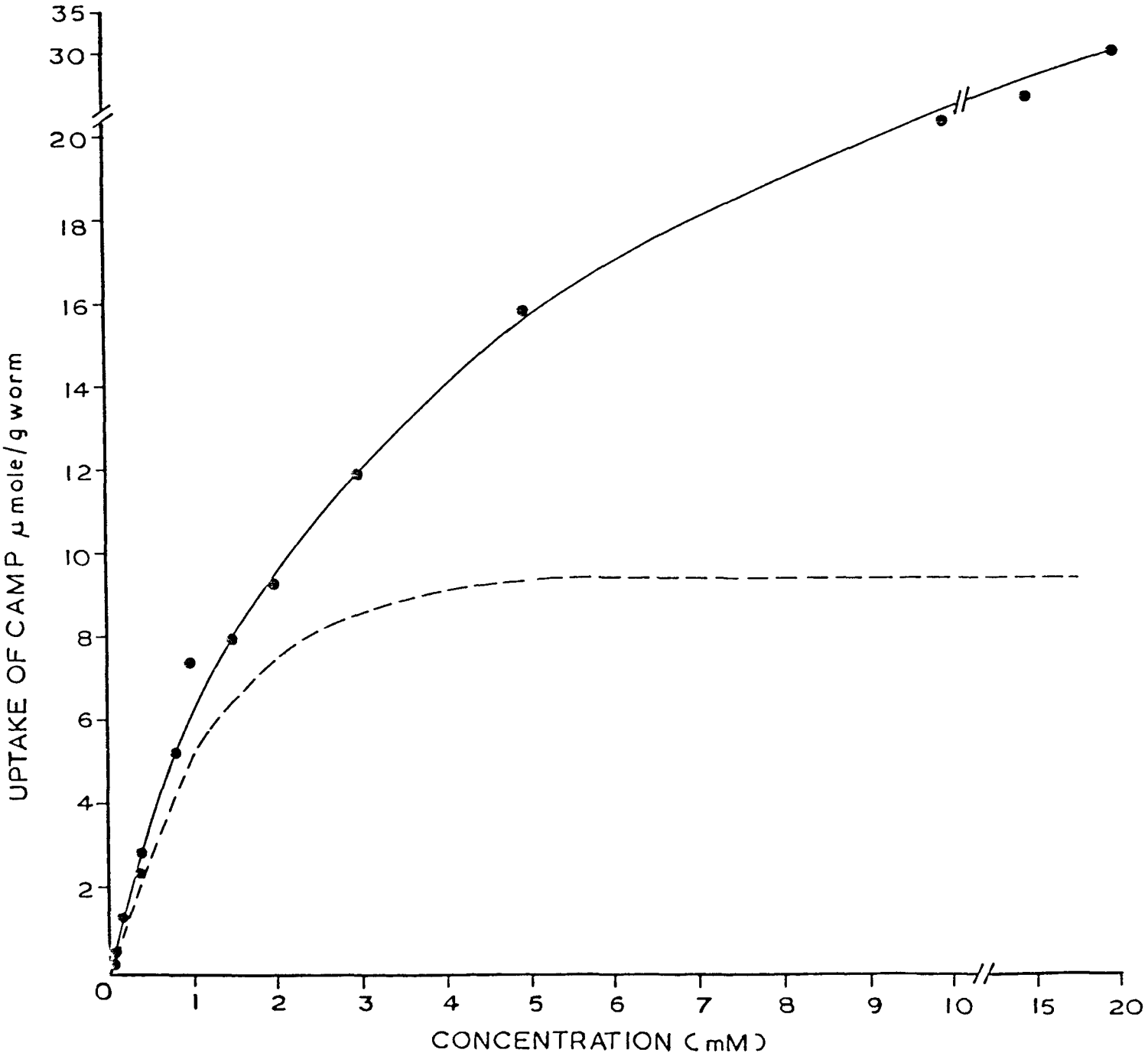

35
30
20
18
16
14
12
10
8
6
4
2
0
UPTAKE OF CAMP μmole/g worm
1
2
3
4
5
6
7
8
9
10
15
20
CONCENTRATION (mM)

Fig. 14. Curve represents double reciprocal plot of Fig. 13.

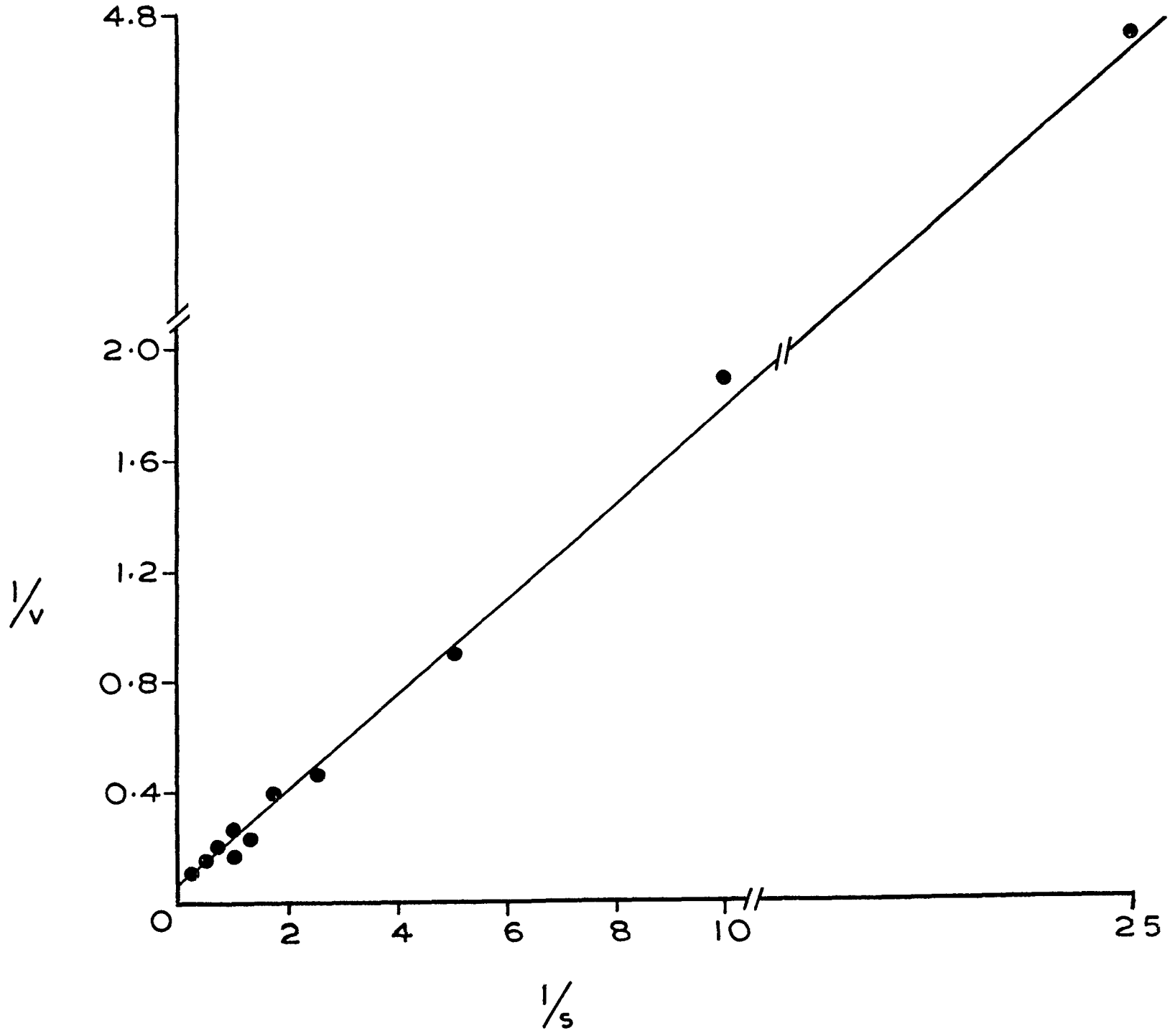
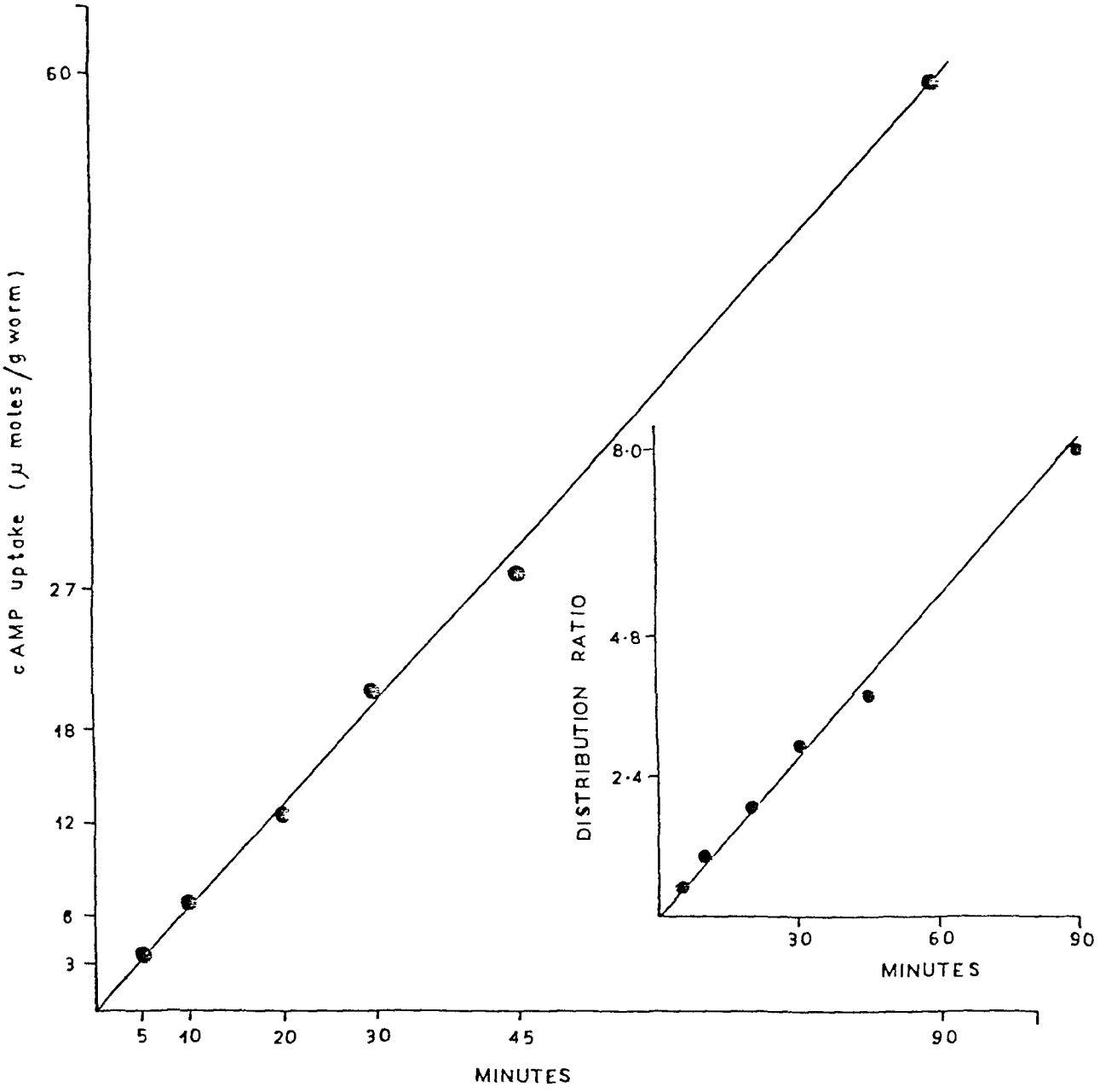

4.8
2·0
1·6
1·2
0·8
0·4
0
2
4
6
8
10
25
1/v
1/s

Fig. 15. Uptake of cAMP by D.viteae as a function of time. Figure in the inset illustrates the relationship between the distribution ratio and the time of incubation.


60
27
18
12
6
3
cAMP uptake (μ moles/g worm)
5
10
20
30
45
90
MINUTES
8·0
4·8
2·4
DISTRIBUTION RATIO
30
60
90
MINUTES

DISCUSSION

The above results (Tables 13 and 14) indicate that *D.viteae* possesses mechanisms for transcuticular absorption of cAMP as well as isoproterenol. Uptake of both the solutes appears to be by mediated transport. The observed inhibition of uptake by lectins (Table 15), which bind different kind of sugar moieties on the surface strongly support this view and indicates that surface constituents (possibly glycoproteins) are involved in the transport of both compounds. It is pertinent to mention that the presence of galactosamine, galactose, glucose/mannose on the surface of the two filariids has also been recorded (Table 17, Section C). *L.carinii*, which absorbs cAMP (Table 13) but not isoproterenol (Table 14), thus differs from *D.viteae*. Uptake of cAMP by *L.carinii* also appears to be a facilitated diffusion process. Inhibition of various lectins (Table 15) again indicates that surface sugar moieties participate in this process.

Kinetics of cAMP uptake in *D.viteae* shows that uptake of cAMP occurs through active transport (Fig. 13) with a maximum rate of 0.672 umole/gm/min. Fig. 13 shows that diffusion is also involved in the transport process that is in between solid line and dotted line.

Thus, the two filariids, D.viteae and L.carinii, differ greatly from each other in the transcuticular absorption of cAMP and isoproterenol. This difference may be attributed to differences in their predilection site necessating metabolic adjustments. D.viteae lives in the subcutaneous tissues and has an anaerobic type of metabolism (Wang and Saz, 1974) whereas L.carinii lives in the pleural cavity and metabolizes sugars as a facultative aerobe (Bueding, 1949). cAMP is known to regulate metabolic activities of various mammalian tissues, including muscle (Jost and Rickenberg, 1971), as well as glycolysis in helminth parasites (Mansour, 1962; Donahue et al., 1981). Isoproteerenol, in turn, regulates the level of cAMP through adenyl cyclase (Jost and Rickenberg, 1971). It appears, therefore, that D.viteae adapts itself to the environment and hence responds better to these regulatory molecules. In contrast, L.carinii, which is bathed in the pleural fluid, does not require such a system.

CONCLUSION

Results described in this chapter clearly indicate towards the occurrence of transcuticular uptake of organic solutes in intestinal as well as tissue dwelling nematodes. Certain salient achievements of the study are as follows:

1. Uptake rate may vary with sex and age of the parasite depending on their need for cellular processes.

2. Such variations may also occur from parasite to parasite. This may be due to adaptation of the parasite relating to their niche.
3. Transport ma y involve any of the mechanism i.e. active, facilitated or simple diffusion. Substances of the environment which may interact with the transporting moieties (like drugs and poisons and lectin etc.) may significantly affect the extent of uptake of the crossing molecule.

SECTION C

LECTINS AS PROBES FOR DETERMINING THE MODE OF ACTION OF ANTIFILARIAL DRUGS ON MICROFILARIAE

RESULTS

General information regarding molecular weight and sugar specificity of various lectins, used in this study, is given in Table 16. When the microfilariae of various species, after half an hour incubation with FITC treated lectins, were examined under flourescent microscope they all were found to be illuminated with characteristic glow, indicating the presence of flouresceinated lectins on the surface of microfilariae.

Quantitative pattern of the binding of these lectins with different kinds of microfilariae has been presented in Table 17. It may be noticed that all the lectins

Table 16 - FITC-lectins*

Lectins	Molecular weight	Sub units	Carbohydrates specificity
Concanavalin-A (Con-A)	110,000	4	α D Mannose, Glucose
Ricinus communis agglutinin (RCA_1)	120,000	4	α D Galactosamine, β D Galactose
Ricin (RCA_2)	60,000	2	α or β D galactose, N-acetyl galactosamine
Carcinoscorpin (CSN)	420,000	2	Sialic acid
Wheat germ agglutinin	23,000	2	N-acetyl D glucosamine

* From the literature supplied by Hy-Gro Chemicals, Calcutta.

Table 17 - Binding capacity of different microfilariae to various lectins

Lectins	p moles/million x 10^5		
	L.carinii	*D.viteae*	*B.malayi*
Con-A	43.4	66.0	188.0
RCA_1	36.0	94.3	45.3
RCA_2	47.0	44.3	190.0
CSN	68.4	47.6	32.0
WGA	806.9	1706.9	92.7

The data are mean of two experiments

regardless of their sugar specificity bind with all the three types of microfilariae; the difference at the quantitative level was, however, recorded from species to species and from one type of lectin to the other. Thus while WGA, was the most reactive lectin for L.carinii and D.viteae, RCA_2 was for B.malayi. Similarly RCA_1 showed preference for unsheathed mf of D.viteae compared to sheathed type of microfilariae, L.carinii and B.malayi. CSN, in contrast, exhibited very poor affinity for either type of the mf. Con-A, indicating for the presence of glucose/mannose, the neutral sugars, occupied a central position regarding intensity of interaction with the microfilariae.

Table 18 gives an account of the effect of antifilarial drugs on the binding capacity of various microfilariae to lectins. Both the drugs nevertheless of the nature of the mf or that of the lectin enhanced the affinity of the former to the latter. Closer analysis of the data however, revealed specific differences among the parasites. Thus whereas D.viteae exhibited significant increase in the binding for Con-A and RCA_2, the other two microfilariae i.e. L.carinii and B.malayi elicited enhanced interaction with WGA. The binding of CSN as a result of drug treatment, on the other hand, was found to be increased with all the three species of mf. RCA_1 in contrast, did not show any significant change.

Table 18 - Effect of antifilarial drugs on the binding of various lectins

Lectins	% change to control					
	L.carinii		*D.viteae*		*B.malayi*	
	DEC	Centperazine	DEC	Centperazine	DEC	Centperazine
Con-A	+ 23.85	- 19.66	+ 26.37	+ 41.77	- 26.95	- 13.05
RCA_1	- 26.24	- 0.28	+ 4.47	+ 7.27	+ 7.58	+ 12.80
RCA_2	+ 8.52	+ 12.78	+ 72.02	+ 76.04	- 1.75	- 1.75
CSN	+ 36.00	+ 44.00	+ 77.58	+ 57.68	+ 76.92	+ 23.08
WGA	+ 20.73	+ 18.26	+ 0.27	- 5.34	+ 38.96	+ 48.78

\+ Increase

\- Decrease

DISCUSSION

Lectins, the carbohydrate binding and cell agglutinating proteins/glycoproteins have found widespread applications in cell biology as macromolecular probes for delineating the architecture of cell surface glycoconjugates (Rapin and Burger, 1974). They bind to sugar molecules much as enzymes combine with their substrates and antibodies combine with their antigens (Lis and Sharon, 1977).

Using lectins as specific markers various investigators have characterized the carbohydrate residues on the surface of a number of parasites. For instance, Simpson and Smithers (1980) were able to quantitate the sugar residues exposed at the surface of adult S.mansoni using radio-isotope labelled lectins. Flourochrome-labelled lectins have also been very useful for this purpose (Bennett and Seed, 1977; Murrell et al., 1978; Stein and Lumsden, 1973; Wilson and Barnes, 1977). Linder and Hudlt (1982) used flouresceinated lectins to determine the exposed and hidden carbohydrates of S.mansoni adult worms.

The use of flouresceinated lectins for determining the sugar moieties on the parasites surface has once again been proven by the present study. The results clearly indicate that the microfilarial surface does not differ very much from species to species regarding the presence

of sugar moieties; it only differs in respect of their quantitative distribution (Table 17). For example, while L.carinii and D.viteae possess maximum number of N-acetyl glucosamine residues, B.malayi has highest density of N-acetylgalactosamine residues on its surface. Such differences may be very important in establishing the parasite against host defence survellience and hence regarding their host specificity. This becomes more clear when the effect of antifilarial drugs are taken into account. According to Schardein et al. (1968), L.carinii microfilariae freely swim in the blood and pass through the liver. However within five minutes of treatment of the host with diethylcarbamazine (DEC), microfilariae are found to be engulfed and accumulated in the liver. This probably is due to the increase of sialic acid and N-acetyl glucosamine type of residues on the parasite's surface (Table 18). It is pertinent to add here that rat hepatocytes have been reported to possess mannose and N-acetyl glucosamine specific lectins (Maynard and Baenziger, 1982). Microfilariae of B.malayi like that of L.carinii also shows the increased density of the above two sugar residues. For D.viteae the increase is found in the amount of Con-A and RCA_2 indicating that for this microfilariae instead of N-acetyl glucosamine the role is probably played by N-acetyl galactosamine, the other type of basic sugar. Though an increase in sialic

acid is also noticed with all the three microfilariae, however, if the net increase in number of acidic and basic residues is taken into account, the balance goes in favour of basic sugars. A simultaneous increase in the binding of two lectins triggered by the drugs may represent "positive co-operativity". Such a phenomenon where the binding of one ligand to one site increases the affinity for the ligand of the neighbouring site, has first been reported for lectins by Thom et al. (1979). It therefore appears that the drug primarily increases the binding of CSN which subsequently induces an enhancement in the binding of basic sugars. It may be inferred from this that in D.viteae mf the sites of N-acetyl galactosamine and glucose/mannose (i.e. the moieties binding to RCA_2 and Con-A) are neighbouring to that of sialic acid (CSN); while for the other two sheathed type of microfilariae i.e. L.carinii and B.malayi the neighbouring residue is N-acetyl glucosamine (WGA). Many more studies are required before anything may be said definitely in this regard. It is however, clear that the trapping of microfilariae by the liver following the drug treatment is possibly due to the increase in the binding sites on the microfilarial surface for acidic and basic residues which provide firm interaction between the mf and liver.

SECTION D

EFFECT OF SUGARS AND DRUGS ON CONCANAVALIN-A BINDING WITH ADULTS DIPETALONENA VITEAE

In the preceding chapter a panel of flouresceinated lectins were used to analyze the carbohydrate moieties present on the microfilarial surface. The effect of filaricides on the interaction of lectins has provided some lead in understanding the possible mechanism of action of these compounds. In this chapter radiolabelled Con-A has been used for analyzing the effect of various agents on the availability of glucose/mannose residues of adult D.viteae.

RESULTS

Table 19 illustrates that Con-A binding increases with increasing concentration of the lectin in the incubation medium. Glucose, mannose and methyl glucose

Table 19 - Con-A binding with adults *D.viteae**

S.No.	Concentration	Binding (n moles/mg x 10^{-6})
1	1 x 10^{-7}M	140.4
2	4 x 10^{-7}M	144.0
3	1 x 10^{-6}M	153.6
4	4 x 10^{-6}M	159.5
5	1 x 10^{-5}M	470.4
6	4 x 10^{-5}M	514.8
7	1 x 10^{-4}M	591.6

* Sp.act. = 5 uCi/4 nmole

Values are mean of two experiments

15-20 mg worms were incubated for 1 hr at 37^{o}C in 1 ml Con-A dissolved in BFM.

exhibited interesting effects on the binding of this lectin (Table 20). These sugars with the living worm while showed poor inhibition, with the dead worms, in contrast, they elicited significant inhibition of binding. Table 21 summarizes the effect of a few anthelmintics on the binding of Con-A with adult D.viteae. It may be noticed that whereas DEC, Centperazine and mebendazole increased the binding from 10-20% only tetramisole increased it by 45%. Not all but nearly half of the lectin taken up by D.viteae may be detached from the parasite by the action of trypsin (24%) followed by triton x-100 (20%) (Table 22).

DISCUSSION

It is clear from the above results that adult worms of D.viteae possess free residues of glucose/mannose on its surface. The evidence that this interaction is a specific type and does not arise because of exchange of radioactivity is provided by the inhibition of the binding by glucose, mannose and methyl glucose (Table 20). Very high inhibition of the binding by these sugars with dead worms compared to that with the living worms may be due to the possibility of the ingestion of the lectin by the living worms (Table 20) but not by the dead worms. Higher uptake of the lectin by the living worms further strengthen this possibility. Though all the anthelmintics examined

Table 20 - Effect of sugars on the binding of Con-A with *D.viteae*

Sugar	Concentration	Inhibition %*	
		Active worms	Dead worms
Glucose	1×10^{-3}M	19.0	70.9
Methyl glucose	1×10^{-3}M	9.5	39.6
Mannose	1×10^{-3}M	17.0	40.3

* Control values = 470.4×10^{-6} n moles/mg for living and 138.4×10^{-6} n moles/mg for dead worms. Values are mean of two experiments.

Table 21 - Effect of drugs on binding of Con-A with *D.viteae*

S.No.		n moles/ mg x 10^{-6}	Increase in binding (%)
1. Control	-	330	-
2. DEC	1 x 10^{-3}M	384	16
3. Centperazine	"	394	18
4. Tetramisole	"	480	45
5. Mebendazole	"	375	13

* Average values of two separate experiments

Table 22 - Extraction of bound Con-A with different agents

S.No.	Activity released (%)
1. Trypsin	24.0
2. Triton x 100	19.6
3. Residue	56.3

for their activity increased the binding of the lectin, tetramiode exhibited highest and significant enhancement of the binding. The increased binding may be due to the damage of the cuticle by the drug. There are numerous reports dealing with the alterations on the cuticle of parasites caused by drugs. For example Gibson et al. (1976) described some specific changes in the cuticle of the microfilariae of Onchocerca volvolus collected from patient after DEC treatment. Similarly Langham and Kramer (1980) have reported that microfilariae and fully developed larvae of O.volvulus showed decrease in motility and subsequently died due to the effect of DEC and that was associated with a randomized loss of darkly refractile nuclei seen by phase-contrast microscopy. In vitro experiments of Piessens and Beldekas (1979) on B.malayi microfilariae showed that DEC increases the adherence of leukocytes to microfilariae, mediated by antibodies reacting to the surface of microfilariae. DEC, mebendazole and levamisole hydrochloride have direct effect on the motility of infective larvae of B.malayi and there was cuticular damage as confirmed by scanning electron microscopic studies of Sim et al. (1983). It may be concluded that drugs directly affect the cuticular surface including carbohydrate moieties of different stages of filariae which may be the initial step in the anthelmintic action because cuticle of the parasite is the first organ to come in contact with the drug.

CHAPTER V

SUMMARY AND CONCLUSION

SECTION A

Isolation, purification and characterization of surface membrane protein from *Ascaridia galli*

Employing papain as the extracting agent, proteins/glycoproteins have been extracted from the surface of intact parasites. The yield of the crude material was approximately 1.5 mg from one gm of worms. The crude extract contained 5.5% carbohydrates showing the presence of glycoproteins. Trypsin and pepsin did not give satisfactory results. As the control set of worms did not excrete any glycoprotein in the medium it was ensured that the extracted glycoproteins/proteins were solely from the surface of *A. galli*. A glycoprotein from the crude extract was purified by affinity chromatography on Agarose-RCA_1. This glycoprotein exhibited single band on polyacrylamide gel electrophoresis and molecular weight as 68,000 on SDS-PAGE. Pure glycoprotein comprised of 10%

carbohydrate and on analysis by HPLC revealed the presence of fucose, galactose, rhamnose and glucosamine. Comparative binding study of crude extract and pure glycoprotein with various organic compounds showed that crude extract did bind with serine, aspartate, glutamate and isoproterenol while purified glycoprotein bound with aspartate and glutamate with increased affinity. Circular dichroism spectra of purified protein in the far UV region showed the peptide backbone and comprised of 2% α-helix. However, addition of glutamate caused the shift of entire spectra from negative to positive side with α-helix 18%.

SECTION B

1. Transcuticular absorption of amino acids by *Ascaridia galli*

Since both oral and anal openings were ligated, the uptake represents transcuticular absorption. It is observed that the unligated worms, in general, exhibit higher uptake of amino acids compared to the ligated ones. Male or female worms, except having a slight difference at the quantitative level, display similar pattern regarding uptake of various amino acids. Thus, parasites of both sexes absorb prline and lysine at highest rate. Serine, leucine and aspartic acid are taken up at moderate rates, while alanine and arginine show poor absorption.

Age of the parasite appears to have a great influence on the absorption of amino acids; young parasites utilize these acids at relatively faster rate. Uptake of proline and serine as a function of their concentration exhibits linearity and does not show saturation at the highest concentration used thereby indicating that these amino acids cross the cuticle by simple diffusion. These amino acids are utilized not only for protein synthesis but also are involved in energy generating process.

2. Transcuticular uptake of methyl glucose by Setaria cervi

Uptake of methyl glucose by S.cervi was found to occur through ingestion as well as through transcuticular absorption. The latter process involved an active transport mechanism showing K_t of transport as 1.67 mM and V_{max} as 3.57 u mole/hr/g. Diffusion did not appear to play a role in the absorption of this sugar. Triphenyltinchloride and p-hydroxymercuribenzoate (pHMB) exhibited non-competitive inhibition while phloridzin acted as a competitive inhibitor. Triphenyltinchloride like other tin compounds inhibited the uptake by impairing the energy generating system of the parasite or by binding at the transport site. pHMB, which mimiced triphenyltinchloride in all respects, also appeared to act by interfering either with the energy metabolism or by binding to the transport site. Phloridzin did not have any effect on the motility of

the parasite whereas triphenyltinchloride and pHMB, at a final concentration of 5×10^{-5}M or higher, exhibited toxicity and apparently killed the worm. 2,4-Dinitrophenol and KCN interfered with the transport of methylglucose where the effects of dinitrophenol were more pronounced. Metabolizable sugars like glucose, mannose and ribose reduced the uptake of methyl glucose while non-metabolizing sugars like fructose and galactose enhanced the influx. These observations clearly indicated that movement of small molecules through cuticle was not limited to intestinal nematodes only but it also occurred in filarial parasites where physiologically significant amount of sugar was taken by active transport mechanism and metabolic inhibitors and inhibitors of energy production caused reversible or irreversible effects depending on their nature.

3. Uptake of adenosine 3',5'-cyclic monophosphate (cAMP) and isoproterenol by filarial parasites

D.viteae has been found to possess mechanisms for transcuticular absorption of cAMP as well as isoproterenol. Uptake of both the solutes appears to be mediated by active transport since the absorption shows an uphill accumulation. Inhibition of uptake by lectins strongly support this view and indicate the involvement of surface constituents (possibly of glycoproteins). However,

L.carinii absorbs only cAMP by facilitated diffusion process. Thus it differs from D.viteae. This difference may be attributed to the difference in their seat of predilection site necessating metabolic adjustments. D.viteae resides in subcutaneous tissues and has anaerobic type of metabolism. In contrast, L.carinii is found in pleural cavity and possesses semiaerobic metabolism.

SECTION C

Lectins as probes for determining the mode of action of antifilarial drugs on microfilariae

D.viteae microfilariae (mf) possess more binding sites for WGA followed by CSN, RCA_1, Con-A and RCA_2. L.carinii mf showed maximum binding with CSN followed by WGA, Con-A, RCA_1 and RCA_2. B.malayi like D.viteae mf also possesses more binding sites for WGA followed by CSN, RCA_2, Con-A and RCA_1. However, these lectins bind only with the surface moieties of mf may not be claimed because there is also the possibility of their interactions with the inner constituents also. Preincubation of antifilarial drugs DEC and centparazine enchance the binding of lectins to the microfilariae specially CSN and WGA. These antifilarial drugs, in fact, expose the binding sites of sugar molecules for the interaction of lectins.

This reflects that administration of drugs in filaria infected patients/rats enchances adhesiveness of the microfilarial surface thereby causing their trapping in the host liver.

SECTION D

Concanavalin A binding with adults D.viteae

Binding of Con-A with adult worms of D.viteae indicates for the presence of glucose/mannose residues on the surface of this filarial parasite. Inhibition of this binding by glucose, mannose and methylglucose support the above view. The inhibition of the binding was more pronounced with dead worms compared to that with the live worms. The reason for this difference may be attributed to the uptake of the lectin by live worms over its binding to the surface. All the anthelmintics used in the study namely, DEC, Centperazine, tetramisole and mebendazole enhanced the binding of the lectin which may be suggestive of the damage caused by the drugs to the filarial surface.

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